Studies on the effect of cysteinyl leukotrienes on human t cell differentiation and function

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STUDIES ON THE EFFECT OF CYSTEINYL LEUKOTRIENES ON HUMAN T CELL DIFFERENTIATION AND FUNCTION

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Abstract

Antigen-specific Th2 cells are central to the pathogenesis of asthma and other allergic diseases. Th2 cells secrete the cytokines IL-4, IL-5 and IL-13, responsible for many of the features of allergic inflammation such as eosinophilia, IgE production and mucus hypersecretion. Cysteinyl leukotrienes (CysLTs) are known potent lipid mediators involved in mucus production, bronchoconstriction and leukocyte migration, and act via the receptors $CysLT_1R$ and $CysLT_2R$. Recently, CysLTs have been implicated in Th2 responses in mouse models, although the exact mechanisms are unclear.

Preliminary microarray studies on polarised human Th1 and Th2 cells suggested a highly selective expression of CYSLTR1 by human Th2 cells. Subsequent RT-PCR analysis confirmed that Th2 cells selectively express CYSLTR1 mRNA at high levels. Calcium signalling experiments revealed that Th2 cells respond selectively to leukotrienes compared to Th1 cells, with a rank order of potency similar to that reported for $CysLT_1R$ (LTD_4>LTC_4>LTE_4). This profile of leukotrienes responsiveness was blocked using the known selective $CysLT_1R$ antagonists MK571, Montelukast and Zafirlukast. Additionally, the LTD_4-induced signalling in Th2 cells is mediated via $CysLT_1R$ coupled to $G_{\alpha q}$ and $G_{\alpha i}$ proteins, which are blocked when using selective $CysLT_1R$ antagonists. Finally, LTD_4 is a potent chemoattractant for Th2 cells, which migrate to LTD_4 at low nanomolar concentrations in a dose-dependent manner. Migration of human Th2 cells towards LTD_4 was inhibited by selective $CysLT_1R$ antagonist MK571. Interestingly, LTD_4 had no significant effect on the proliferation or differentiation of Th2 cells, or on the expression of Th2-specific cytokines. Microarray studies on LTD_4-treated Th2 cells identified key immediate early genes that were upregulated after 30 minutes of treatment, and that were differentially expressed compared to untreated cells.

This is the first report that human T cell subsets express functional $CysLT_1R$ and important implications for our understanding of the anti-inflammatory effect mechanism of $CysLT_1R$ antagonists.

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Abbreviations

5-HETE	5(S)- hydroxy-8,11,14-cis-6-trans-eicosatetraenoic acid
5-HPETE	5(S)- hydroperoxy-8,11,14-cis-6-trans-eicosatetraenoic acid
5-LO	5-Lipoxygenase
7TM	Seven transmembrane
β-gal	β-galactosidase
AA	Arachidonic acid
AHR	Airway hyperresponsiveness
ANOVA	Analysis-of-variance
AP-1	Activator protein-1
ATP	Adenosine 5’ –triphosphate
BAL	Bronchoalveolar lavage
BALF	Broncho Alveolar Lavage Fluid
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BATF	Basic leucine zipper transcription factor
cAMP	Adenosine 3’, 5’ –cyclic monophosphate
COPD	Chronic obstructive pulmonary disease
COX	Cyclo-oxygenase
$cPLA_2$	Cytosolic phospholipase A_2
CysLT	Cysteinyl leukotriene
DAG	Diacylglycerol
DC	Dendritic cell
DH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
EC50	The molar concentration of an agonist required to achieve 50 % of maximum effect
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EGTA	ethylene glycol tetra-acetic acid
EIA	Exercise-induced asthma
EP1	Prostaglandin E receptor
EPAS1	Endothelial PAS domain-containing protein 1
ERK	Extracellular signal-regulated kinase
FACS	Fluorescent activated cell sorting
FCS	Fetal calf serum
FDR	False discovery rate
FLAP	5-Lipoxigenase activating protein
FLIPR	Fluorescence Imagine Plate Reader
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA	Trans-acting T cell-specific transcription factor
GDP	Guanosine Dinucleotide phosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HBSS	Hank's Buffered Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferon
Ig	Imunoglobulin
IL	Interleukin
IP_3	Inositol triphosphate
IP-10	Interferon gamma-induced protein
Jnk	c-Jun NH2 terminal kinase

LT	Leukotriene
LTα	Lymphotoxin-α
LTA_4	Leukotriene A_4, 5, 6-epoxy- 7,9-trans- 11, 14-cis-eicosatetraenoic acid
LTB_4	Leukotriene B_4, 5(S),12(R)-dihydroxy- 6,14-cis-8,10-trans-eicosatetraenoic acid
LTC_4	Leukotriene C_4, 5(S)-hydroxy-6 (R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTD_4	Leukotriene D_4, 5(S)-hydroxy-6 (R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTE_4	Leukotriene E_4, 5(S)-hydroxy-6 (R)-S-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTRA	Leukotriene receptor antagonist
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MIP-2	Macrophage inflammatory protein 2
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κ B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-Buffered Saline
PCA	Principal component analysis
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PI-3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol (4,5)-bisphosphate
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PTX	*Bordetella pertussis* toxin
RIN	RNA integrity number

ROS	Proto-oncogene tyrosine-protein kinase
RUNX1	Runt-related transcription factor 1
S1P	Sphingosine-1-phosphate
SDF-1α	Stromal cell-derived factor 1 (also known as CXCL12)
SERCA	Sarcoendoplasmic reticulum calcium transport ATPase
SLE	Systemic lupus erythematosus
SRS-A	Slow reacting substance of anaphylaxis
STAT	Signal Transducers and Activators of Transcription
T-bet	T-box expressed in T cells
TBS	Tris-buffered saline
TCR	T cell receptor
TFN-α	Tumor necrosis factor – α
THP	Thapsigargin
TLR	Toll-like receptor
TXA2	Thromboxane A2
UDP	Uridine diphosphate
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor

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CHAPTER 1
INTRODUCTION

Chapter 1 Introduction

1.1 Asthma and the immune response

1.1.1 Asthma

Asthma is an immunological, inflammatory and chronic disorder of the conducting airways, which contract inappropriately, spontaneously, and in response to a wide range of endogenous and exogenous stimuli, leading to variable airway obstruction in association with airway hyper-responsiveness (AHR). Asthma has dramatically increased in prevalence in the past two decades and is continuing to do so, with an estimated 235 million people affected worldwide (WHO), including 5.4 million people in the UK (Asthma UK estimate, 15th Feb 2011), with 1.1 million of these being children, amongst whom asthma is the most common chronic disease (Asthma UK estimate). Asthma is characterized by influx to the lung of lymphocytes and eosinophils, airway hypersensitivity to irritant stimuli, and mucus hypersecretion, resulting in airway obstruction (Wills-Karp 1999) and symptoms of wheezing, coughing and difficulty in breathing. It is believed to be Th2 lymphocyte-mediated and, in the case of allergic asthma, is caused by dysregulated immune responses in the respiratory mucosa, following inhalation of a particular irritable substance known as an allergen. These allergens vary in nature; they can be indoor allergens (such as house dust mite), outdoor allergens (pollen), but also tobacco smoke particles or even chemical irritants (Chilmonczyk et al. 1993; Salter 1866). Cold air and exercise have also been reported to induce asthma exacerbations, in the case of exercise-induced asthma (Hallstrand et al. 2005a; Hallstrand et al. 2005b). Long-term asthma results in hypertrophy and hyperplasia of the smooth muscles and deposition of fibrous tissue in the lamina

propria. These result in "airway remodelling", which sometimes involve irreversible loss of lung function (Bousquet et al. 2000).

Although asthma development is multifactorial, Th2-type cytokines such as IL-4, IL-5, IL-13, and IL-9, and Th2-promoting factors including chemokine receptors CCR4 and CCR8 and the transcription factor GATA-3 are all associated with the disease (Mowen and Glimcher 2004; Panina-Bordignon et al. 2001; Ray and Cohn 1999).

1.1.2 Asthma phenotypes

Airway inflammation is central to disease pathophysiology and relates to airway dysfunction partly through the release of potent inflammatory mediators and partly through remodelling of the airway wall. As the disease becomes more severe, the airway becomes more susceptible to a wide range of environmental irritants and has an altered repair response with secretion of growth factors that induce mucus secretion, smooth muscle proliferation, angiogenesis, and fibrosis and nerve proliferation (Holgate 2012). It is believed that combinations of these processes are what shape the different asthma phenotypes and how they respond to treatment (Holgate 2012).

Most asthma begins in childhood in association with sensitization of the airways to common aeroallergens, particularly those derived from house dust mites, cockroaches, animal dander, fungi and pollens. However, several asthma phenotypes have been identified and these come under two main categories: Th2-associated and non-Th2-associated asthma. The concept of Th2-associated asthma has been around for many years and been strongly linked to atopy or allergy, type I hypersensitivity reactions,

eosinophilic inflammation and response to corticosteroids (Holgate 2012; S. E. Wenzel 2012b).

Three phenotypes have been identified within the Th2-associated umbrella of asthma: early-onset allergic Th2 asthma, late-onset persistent eosinophilic asthma and exercise-induced asthma (S. E. Wenzel 2012b).

Early-onset asthma is believed to occur in early childhood and is linked to atopy or allergy. In particular, Th2-allergic asthma is associated with high levels of eosinophils, mast cells, total and specific IgE and Th2 cytokines. The main therapy for this type of asthma remains corticosteroids, with Th2 pathway-targeted therapies also having some positive effects, such as antibodies to IgE, IL-4Rα and IL-13 (S. E. Wenzel 2012b).

Late-onset persistent eosinophilic asthma is characterized by the presence of higher levels of eosinophils in the sputum and symptoms can currently be maintained using corticosteroids. As patients suffering from this type of asthma are usually observed to have high levels of cysteinyl leukotrienes, anti-leukotrienes agents have been shown to be beneficial to lung function and symptoms. Recently anti-IL-5 therapy has been found to be effective in diminishing blood and lung eosinophils, and decreasing exacerbations (Nair et al. 2009; S. E. Wenzel 2012b).

Finally exercise-induced asthma (EIA) refers to asthma whose symptoms are experienced primarily after exercise. These symptoms involve mild asthma and reactive bronchoconstriction in response to sustained exercise and frequently occur in dry and cold weather conditions. EIA has been associated with reports of levels of eosinophilia, high levels of mast cells and release of their mediators but these reports have been contradictory (Hallstrand et al. 2005a; Karjalainen et al. 2000). A good treatment response in EIA has been seen with the use of anti-cysteinyl leukotriene

agents, although recent studies have suggested that monoclonal antibody blockade IL-9 may also be a potential treatment (S. E. Wenzel 2012b).

Non-Th2-associated asthma represents approximately 50% of all asthma and is less understood. So far this subgroup comprises of obesity-related asthma and neutrophilic asthma.

As the name suggests, obesity-related asthma is mainly driven by obesity and its symptoms. Although it is difficult to diagnose asthmatic symptoms in obese patients, high expression levels of TNF-α, IL-6 and leptins have been identified. No specific treatment or biomarkers have been identified for this phenotype.

In neutrophilic asthma, high levels of neutrophilia are seen in the lung and are associated with lower lung function, more trapping of air and thicker airway walls. Specific treatments remain to be identified as corticosteroids and anti-TNF-α therapies have shown limited effectiveness (Green et al. 2002; S. E. Wenzel 2012b).

1.1.3 The Th2 response

The lung inflammatory response and generation of Th2 response are initiated in association with the airway epithelium and underlying mucosa in which reside professional antigen-presenting cells (APCs) called dendritic cells (DCs). DCs have the ability to take up allergens, process them into small peptides and present them via MHC Class II for recognition by T cell receptors (Holgate 2012). Following antigen uptake, antigen-loaded DCs undergo maturation and migrate from the peripheral tissue to the draining lymph nodes. Once in the lymph node, the antigen-loaded DCs prepare for interaction with lymph node-homing T cells by lining up along the high endothelial venules. The DCs prepare for the priming of T cells in the lymph nodes. The

initial encounter between the DC and the T cell leads to the upregulation of T-cell activation markers such as CD44 and CD69 and the secretion of cytokines such as IL-2. The third step of the interaction between the DC and T cell occurring within the lymph node results in increased T cell motility as well as rapid STAT-independent proliferation. DC-activated antigen-specific CD4+ T cells then migrate to the infected peripheral tissues where they encounter IL-4 and differentiate into antigen-specific Th2 cells, secreting high levels of effector cytokines in a STAT6-dependent manner. The source of IL-4 has been the subject of debate and there is now increasing evidence that this may come from basophils (Holgate 2012), as discussed later on. These Th2-specific cytokines such as IL-4, IL-13, IL-9 and IL-5 are essential players in the lungs of asthmatic subjects.

IL-4 signals via a specific IL-4 cell-surface receptor composed of the IL-4Rα chain and the γ common chain, and has been shown to be vital for the regulation of growth, differentiation, activation, and function of B cells (Tangye et al. 2002). IL-4 has been found to increase the expression of the antigen-presenting proteins, MHC class II molecules, on B cells, resulting in increased allergen presentation capacity to Th2 cells (Hamid and Tulic 2009; Seder and Paul 1994). IL-4 has also been shown to promote expression of VCAM-1 on endothelium (Schleimer et al. 1992), thereby allowing for recruitment of eosinophils and other inflammatory cells such as T cells, monocytes, and basophils from the blood into the sites of inflammation (Hamid and Tulic 2009). IL-4 is also involved in inducing IgE isotype class switching by B cells. Following class switching, IL-4 is known to potentiate IgE production and enhance the IgE-mediated response by upregulating IgE receptors on inflammatory cells within the airway (Vercelli et al. 1988).

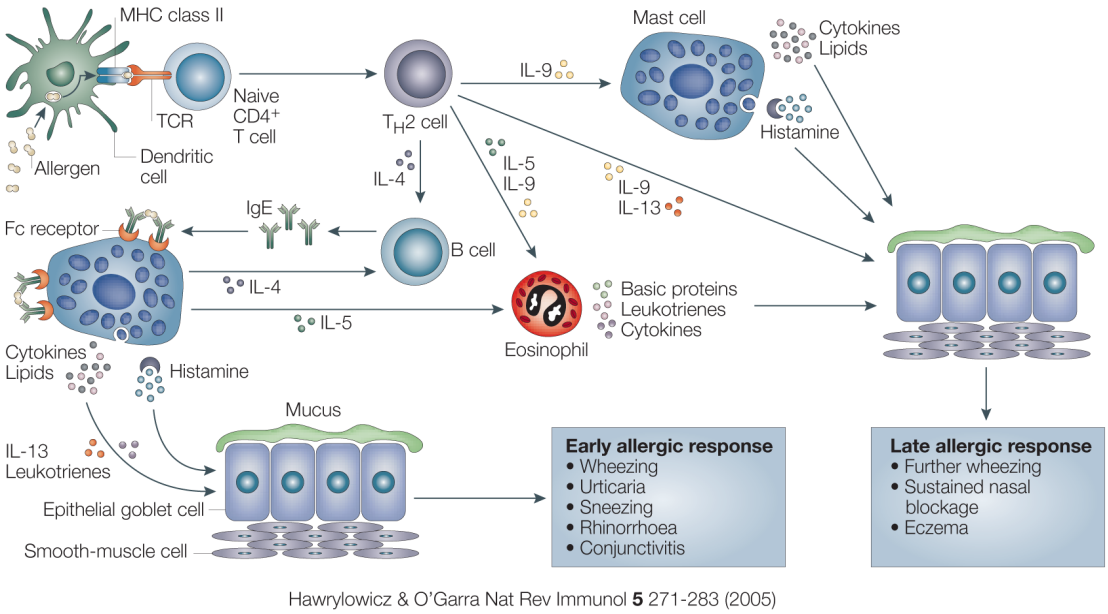
IL-13 shares 70% sequence homology with IL-4, and binds a heterodimer composed of the IL-4Rα chain and an IL-13Rα chain (Mitchell et al. 2010; Oh et al. 2010). Like IL-4, IL-13 is produced by Th2 cells and both cytokines have some overlap in their functions. Similarly to IL-4, overexpression of IL-13 within the lungs has been shown to lead to IgE production, inflammation, mucus hypersecretion, eosinophilia, and upregulation of VCAM-1 (Wills-Karp et al. 1998).

IL-9 is also relevant to IgE-dependent host responses and its expression is regulated by several mediators, in particular IL-2, which stimulates its production. Although IL-9 is produced by several cell types, including mast cells, eosinophils, and neutrophils (Hultner et al. 2000; McNamara et al. 2004; Shimbara et al. 2000), the major sources of IL-9 are Th lymphocytes. Two independent groups have demonstrated that naive CD4+ T cells primed in the combination of TGF-β and IL-4 or Th2 cells additionally cultured in TGF-β can produce high levels of IL-9 with reduced expression of other lineage-specific cytokines and transcription factors and promote the development of a CD4+ T cell subset that preferentially produces IL-9, and which has therefore been termed Th9 cells (Dardalhon et al. 2008; Veldhoen et al. 2008). *In vitro*, IL-9 has been shown to enhance IL-4-mediated IgE production by both human and murine B cells (Dugas et al. 1993).

IL-5 is the most important Th2 cytokine associated with eosinophils, and has been shown to regulate most aspects of eosinophil behavior including eosinophil growth, maturation, differentiation, survival, and activation. IL-5 is known to play a central role in accumulation and activation of eosinophils in the lungs. Although IL-5 is produced by T helper cells, cytotoxic T lymphocytes, and mast cells, eosinophils are the

predominant sources of this cytokine and it has been shown to act as a potent eosinophil chemoattractant. IL-5 also upregulates integrin receptor expression on eosinophils, thereby promoting adherence of eosinophils to VCAM-expressing endothelial cells and eosinophil accumulation (Hamid and Tulic 2009; Larche et al. 2003).

IL-4, IL-9 and IL-13 are therefore particularly critical for their role in enhancing IgE production from B cells, leading to IgE and Fc receptor cross-linking on the surface of mast cells, resulting in their degranulation, and the subsequent release of inflammatory mediators such as histamine, prostaglandin D2 and cysteinyl leukotrienes (see figure 1.1); while IL-5 is involved in the recruitment and activation of eosinophils. The release of inflammatory cytokines and mediators by mast cells results in the known features of asthma, such as bronchoconstriction, eosinophil and basophil infiltration, mucus hypersecretion, bronchial remodelling, and airway hyper-responsiveness (AHR) ((Holgate 2008; Kay 2005; Larche et al. 2003; Umetsu and DeKruyff 2006). The inflammatory process is then maintained by the recruitment of more inflammatory cells into the lungs, such as eosinophils, basophils, neutrophils and lymphocytes (Djukanovic et al. 1990).



Hawrylowicz & O'Garra Nat Rev Immunol **5** 271-283 (2005)

Figure 1.1 Representation of the Th2 response during allergic inflammation (Hawrylowicz and O'Garra 2005).

1.1.4 Th1 and Th2 cells

The Th1/Th2 cell hypothesis emerged in the late 1980s, stemming from observations in mice of two subtypes of T-helper cells, originally characterised by the cytokine profile they secreted and the functions they carried out (Mosmann et al. 1986). This hypothesis was adapted to human immunity, with Th1- and Th2-helper cells directing different immune response pathways. Since the original findings by Mosmann and Coffman in 1986, the study of Th1 and Th2 CD4+ T cells has become a very popular field of research and its progress has been extensively reviewed over the last few years (Koppelman and Nawijn 2011; Lloyd and Hessel 2010; Mosmann and Coffman 1989a, 1989b; Oliphant et al. 2011).

It is thought that, upon leaving the thymus, all $\alpha\beta$ CD4+ T cells have a naïve phenotype. In order to differentiate into effector cells and carry out their effector functions, naive cells require the appropriate activation signals and cytokine milieu. Type 1 T helper cells (Th1) produce interferon-gamma (IFN-γ), interleukin (IL)-2, and lymphotoxin-α (LTα) (also known as tumour necrosis factor (TNF)-β), which promote inflammatory and cellular immune responses. By contrast, type 2 Th cells (Th2) produce IL-4, IL-5, IL-9, IL-13, IL-6 and IL-10, and are necessary for inducing humoral response to combat parasitic helminths by inducing strong IgE antibody production by B cells, eosinophil activation, and activation of various inflammatory cells such as mast cells and basophils (Romagnani 1999).

The two factors thought to be the most potent in influencing Th1 and Th2 differentiation are IL-12 and IL-4, respectively (Seder et al. 1992). IL-12 induces phosphorylation of STAT4 in differentiating Th1 (Bacon et al. 1995; Szabo et al. 1995), whereas IL-4 activates STAT6 in Th2 cells (Hou et al. 1994). In mice deficient in Stat-4

and Stat-6, Th1 and Th2 development, respectively, were impaired (Kaplan et al. 1996; Thierfelder et al. 1996). Controlling the development and maintaining a balance between Th1 and Th2 cells is crucial as it is believed that excessive Th1 cytokine production is associated with autoimmune diseases and excessive Th2 cytokine production with type 1 hypersensitivity disorders, such as asthma, eczema and allergy (Sallusto et al. 1998). Indeed, in addition to playing different roles in protecting the immune system, polarized Th1 and Th2 cells responses are also responsible for different types of immunopathological reactions (Romagnani 1999, 2000a, 2000b).

1.1.5 Th1 cell differentiation

The important factors in the differentiation of Th1 cells are STAT1 and STAT4, as STAT1 receives signals from IFNs and STAT4 from IL-12. Both STAT1 and STAT4 have the ability to induce T-bet, which in turn can promote IFN-γ production and repress IL-4 transcription with the help of HLX, RUNX3 and ETS family members (Djuretic et al. 2007; Hwang et al. 2005). It has been shown IFN-γ is involved in a positive feedback loop in which IFN-γ can act via T-bet to induce more IFN-γ, including in monocytes, macrophages, dendritic cells and B cells, and this is done through the activation of STAT1 by IFN-γ (Szabo et al. 2000).

IFN-γ has also been reported to positively regulate STAT4, in contrast to IL-4 and GATA-3, which negatively regulate STAT4. Once activated, STAT4 can directly induce IFN-γ production and expression of 12Rβ2 and T-bet during Th1 differentiation. The role of T-bet is also to antagonise Th2 differentiation by inhibiting the function of GATA-3. Some studies have however shown that it is possible for Th1 differentiation to occur without T-bet or STAT4 (Kanno et al. 2012; K. Zhu et al. 2010).

1.1.6 Th2 cell differentiation

CD4+ T cell differentiation is down to three essential signals: TCR engagement, appropriate co-stimulation and cytokine receptor ligation. The first signals thought to induce CD4+ T cells to follow a Th2 differentiation path are T-cell receptor engagement, NFAT and GATA-3. Studies in naïve transgenic CD4+ T cells have reported that a low-strength TCR signal (0.5pg/ml of peptide) is preferable to induce IL-4 secretion. This led to a weak and transient ERK activation and GATA-3 stabilization, triggering the activation of *il4*. This also allowed for bypassing the requirement for exogenous IL-4, by inducing IL-2, which in turn fed back in an autocrine manner and activated STAT5. In contrast, higher doses (over 0.5 μg/ml) have been shown to induce IFN-γ production (Constant et al. 1995; Hosken et al. 1995).

In order to provide this low TCR signal, the question remains as to which cells are responsible for antigen presentation and providing the signals. There has been a lot of speculation on the role of dendritic cells (DCs) in providing that signal to Th2 cells and acting as both the primary antigen-processing and antigen-presenting cells, using a separate source of IL-4. More recently, several groups have reported that basophils could have the ability to single-handedly induce Th2 cell differentiation, by providing antigen-presenting and antigen-priming capacity as well as the IL-4 signal. There have been contradicting reports on the role of basophils and DCs as the prime T cell differentiation inducer and clear roles remain to be defined (Okoye and Wilson 2011).

Co-stimulatory signals are also important for T cell differentiation, as antigen presentation via MHC II alone is usually not enough. Co-stimulation generally occurs via CD28, ICOS, CTLA-4 and PD-1 on the surface of the T cell and B7 molecules on the

surface of the antigen-presenting cell, following synapse formation between the two cells (Okoye and Wilson 2011).

The third signal essential for differentiation to occur is the right cytokine environment. As mentioned earlier, exogenous IL-4 has traditionally been known for its involvement in Th2 cell differentiation. However IL-4-independent pathways have recently been identified using cytokines such as IL-25, IL-33 and TSLP (Okoye and Wilson 2011).

The differentiation of Th2 cells usually occurs when a naïve T cell is activated by an antigen on the surface of an APC in the presence of IL-4. The IL-4 receptor is composed of the common cytokine receptor gamma subunit and the IL-4Rα chain. IL-4R is expressed at low levels on the surface of naïve CD4+ T cells, but its levels of expression increase upon antigen challenge. The IL-4 binding to the IL-4R results in the recruitment of Signal Transducer and Activator of Transcription 6 (STAT6) (Ansel et al. 2006; Kanno et al. 2012). STATS are DNA-binding transcription factors that induce the transcription of their target genes by recognizing specific DNA consensus sequences (O'Shea et al. 2011). Following recruitment, STAT6 becomes phosphorylated, then dimerizes, and translocates to the nucleus where it can activate transcription of Th2 cell specific genes (Nelms et al. 1999). Genome analysis studies in humans have shown that IL-4-induced regulation of gene transcription is highly dynamic, as only a handful of genes differentially regulated within the first few hours remain differentially expressed at 72 hours (Elo et al. 2010; O'Shea et al. 2011).

There are several transcription factors associated with establishing the Th2 cell phenotype. STAT6 is a major, direct contributor to the Th2 transcriptional profile and induces the expression of Th2 cell master regulator GATA-3 (Ouyang et al. 2000). Other transcription factors induced upon TCR stimulation include NFAT family members,

IRF4, Jun family members, and c-MAF (Ansel et al. 2006; G. R. Lee et al. 2006; K. M. Murphy and Reiner 2002). Of the newly identified STAT6 targets, the three transcription factors RUNX1, EPAS1 and BATF were suggested to play a role in promoting the Th2 cell phenotype by regulating and amplifying Th2 differentiation (O'Shea et al. 2011).

Though particular cytokines are thought to be promoters of specific T cell lineages, a differentiating T cell receives multiple cytokine signals. IL-4 and STAT6 clearly play a dominant role in Th2 cell differentiation. Studies in transgenic mice expressing IL-4 or constitutively active STAT6 are characterized by the development of spontaneous allergic inflammation (Sehra et al. 2008; Tepper et al. 1990). Development of allergic disease is dependent on IL-4 since allergic inflammation is diminished in mice deficient in IL-4 or STAT6 (Akimoto et al. 1998; Brusselle et al. 1995; Kuperman et al. 1998; Sehra et al. 2008).

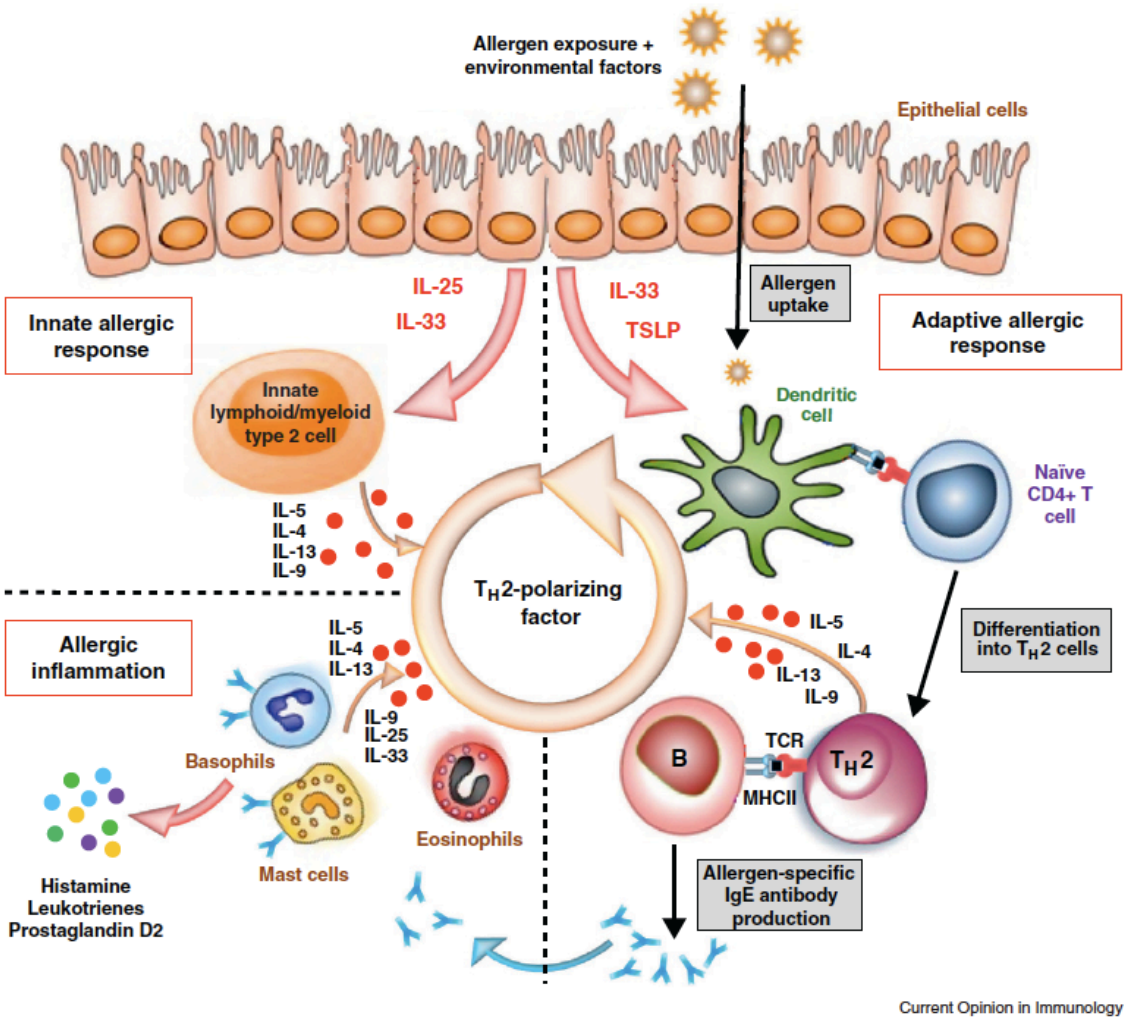


Figure 1.2 Representation of pathogenic processes and Th2 differentiation in allergic disease. Taken from (Wambre et al. 2012).

1.1.7 Other T cell subsets

In recent years, we have attained a much more detailed understanding of CD4+ T cell differentiation, functions, and gene expression profiles, which has led to the identification of additional subtypes. The lineage-specific transcription factor model has been extended and new transcription factors have been identified.

As described earlier on, CD4+ T cells rapidly undergo programmed differentiation following the engagement of the T-cell receptor with peptide-MHC-II complex processed by the antigen-presenting cell (APC), which results in highly polarised immune responses, particularly T cell-specific cytokines and chemokines secretion (Z. Li et al. 2011b; Okoye and Wilson 2011).

It is now evident that naïve CD4+ T cells can differentiate into at least five functionally distinct subsets including Th1, Th2, Treg, Th17 and Tfh (Hirahara et al. 2011; Sakaguchi et al. 2008; Szabo et al. 2003; J. Zhu and Paul 2008).

Differentiation of naïve CD4+ T cells into a particular lineage depends highly on their exposure to antigen, the co-stimulatory molecules expressed and the cytokine environment (Glimcher and Murphy 2000). An imbalance or dysregulation in specific cytokine production in effector T cells can lead to both tissue destruction and chronic inflammation for Th1, and asthma and allergy for Th2 (Hirahara et al. 2011; Mosmann and Coffman 1989a).

Briefly, Th1 cells regulate cell-mediated immunity against pathogen and protect the host against intracellular infections including viruses. Th1 cells produce IL-2 and IFN-y under the transcription regulator T-bet (Mosmann and Coffman 1989a). On the other hand, Th2 cells mediate host defence against parasitic infections such as helminths and produce IL-4, IL-5, IL-13 and IL-9 under the transcription regulator GATA-3.

Regulatory T cells, or Tregs, consist of another CD4+ T cell lineage and comprise of nTregs (thymic natural) and iTregs (peripherally induced). These cells have essential immunosuppressive functions and express IL-10 and TGF-β under the master regulator transcription factor FoxP3 (Zheng and Rudensky 2007). Both natural and induced Tregs are involved in maintaining peripheral tolerance, preventing autoimmunity and inflammatory immune responses.

More recently, the discovery of IL-17-producing CD4+ T cells, or Th17 cells, renewed the interest in T cell differentiation (Weaver et al. 2006). Th17 cells express a splice variant of the retinoid orphan receptor-c, designated RORyt, and produce distinct effector cytokines, including IL-17 (or IL-17A), IL-17F and IL-6 (Weaver et al. 2006). Th17 cells seem to originate from a pathway different to Th1 and Th2 cells, however, it resembles Th1 cells in some ways, which has caused confusion, particularly on the role of Th1 cells in autoimmunity. Th17 cells contribute to host defence against extracellular bacteria and fungi and have also been implicated in the pathology of several autoimmune and inflammatory diseases such as experimental autoimmune encephalomyelitis (EAE) (Becher and Segal 2011).

Following Th17 cells, other lineages have also been identified including Th9 and Th22, which produce their namesake cytokines IL-9 and IL-22, respectively. Th9 cells have been characterized as yet another novel and distinct population amongst the other T helper populations. Th9 cells were first identified as a Th2 sub-population which produced large quantities of the Th2-specific cytokine IL-9, and which could be induced by TGF-β (Neill and McKenzie 2010). Recently, a distinct subset of human skin-homing memory T cells has been shown to produce IL-22, but neither IL-17 nor IFN-γ (Duhen et al. 2009; Trifari et al. 2009). Differentiation of IL-22 producing T cells, now named Th22

cells, can be promoted by the presence of IL-6 and TNF-α or by the presence of plasmacytoid dendritic cells. The human Th22 cell population has been shown to co-express the chemokine receptor CCR6 and the skin-homing receptors CCR4 and CCR10, suggesting a potential role in skin homeostasis and pathology (Duhen et al. 2009; Trifari et al. 2009).

Follicular T helper (Tfh) cells have also been identified as a separate lineage of Th cells. These cells are defined by their expression of Bcl6 and production of IL-21 and surface molecules PD-1, CXCR5 and ICOS. Tfh refers to cells residing in germinal centres of lymphoid centres, in proximity to B cells. Tfh cells are thought to help B cells by promoting B cell class switching and specialize in helping B cells make antibody responses (Wambre et al. 2012).

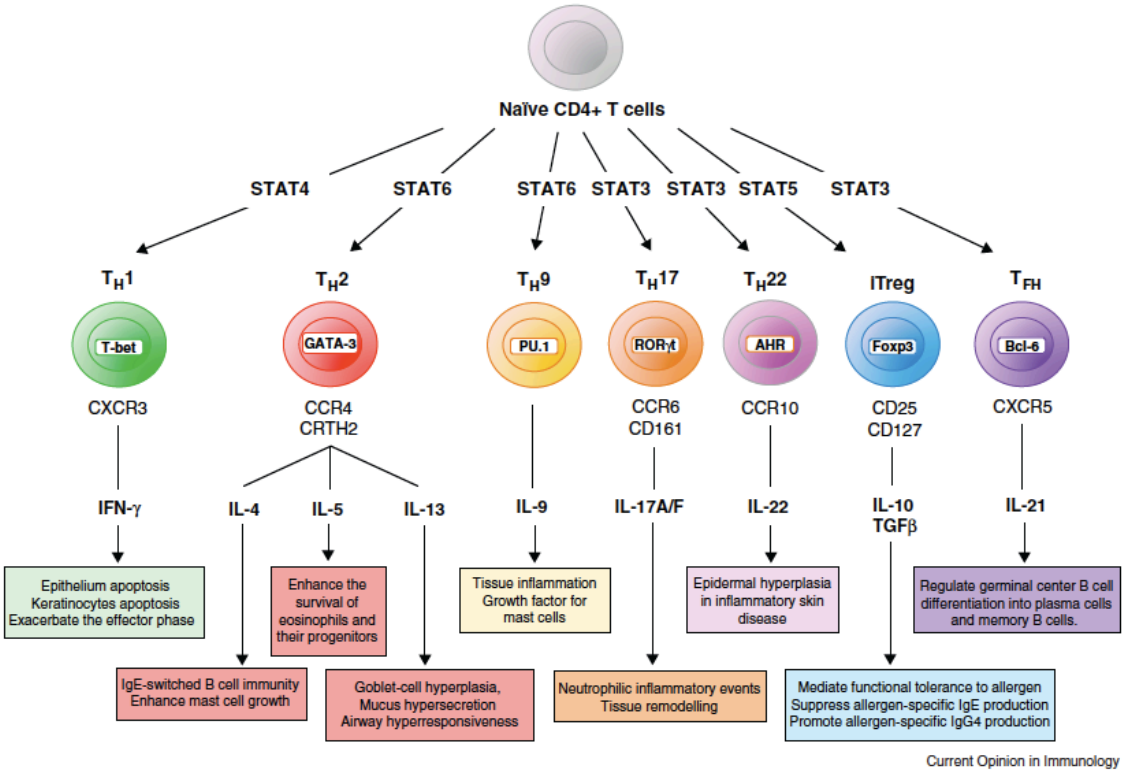


Figure 1.3 Representation of different T cell subsets. This shows the T cell subsets as they are known so far, including their characteristic transcription factors, surface markers, effector cytokines, and other markers (Wambre et al. 2012).

1.1.8 T cell plasticity

There is increasing evidence that the T-helper cell subsets display some plasticity in their phenotypes. Many factors influence the cell fate to become a Th1 or a Th2 cells, such as signal transducer and activator of transcription (STATs), antigen dose, co-stimulatory molecules, genetic modifiers and non-cytokine agents. However how each signalling molecule influences the process of differentiation remains controversial.

A genome-wide analysis study compared patterns of epigenetic modifications in a variety of CD4+ T cells including naïve T cells, Th1, Th2, Th17 and Tregs and found that bivalent permissive and suppressive forms of histone modifications were found at *Tbx21* in all non-Th1 cells. *Tbx21* is the gene locus for T-bet and these results suggested that all non-Th1 differentiated cells retained the capacity to express T-bet, including Th2 cells. These observations were confirmed in Th17 and nTregs, which have been shown to express T-bet and IFN-γ when stimulated through their T cell receptor and in the presence of IL-12 (Y. K. Lee et al. 2009; Wei et al. 2009).

More recently, Hegazy *et al*. showed that Th2 cells were capable of upregulating T-bet and producing IFN-γ following LCMV infection. This was shown only in Th2 cells bearing a TCR specific for the LCMV epitope GP_{61-68} and differentiated for 2-3 weeks *in vitro*. LCMV-induced cytokines IL-12, IFN-γ and IFN-α and IFN-β were also essential to the conversion of Th2 to Th1 cells. Interestingly, GATA-3 in these cells was only modestly suppressed, creating GATA-3^+ T-bet^+ 'Th2+1' cells, which still have the ability to produce IL-4 (Hegazy et al. 2010).

Several studies on Tregs have reported that these cells are able to use CD4+ effector cell-associated transcription factors in order to restore or maintain immune

homeostasis during Th1, Th2 or Th17 immune responses (Campbell and Koch 2011; L. Li and Boussiotis 2011). Indeed, in response to IFN-γ, FoxP3+ Tregs are able to upregulate Th1-specific transcription factor T-bet. In turn, T-bet then induces the expression of CXCR3 on Tregs, which leads to the mobilization of T-bet+ Treg cells at sites of Th1 inflammation. Similar findings have been found with IRF4, a transcription factor for Th2 cells, and mice in which Irf4 has been deleted from FoxP3+ Tregs develop lymphoproliferative diseases associated with an increase in IL-4 and IL-5-producing CD4+ T cells (L. Li and Boussiotis 2011; Zheng et al. 2009).

Additional studies have also shown that Tregs can convert into a Th17 phenotype when induced by the appropriate inflammatory stimuli. It has been shown that under certain conditions, FoxP3 is able to physically bind to RORγt (which are both expressed in Treg cells) and inhibit the transcriptional activity of RORγt, thereby blocking IL-17 production. However it has recently been demonstrated that Tregs can be converted into IL-17+ Tregs if in the presence of antigen-presenting cells and CD4+ T cells. In humans, antigen-presenting cells and the presence of IL-2 or IL-15 as well as enhancement by IL-1, IL-23 and IL-21, have been shown to be the right conditions for IL-17+ Tregs (L. Li and Boussiotis 2011; Voo et al. 2009).

These few studies represent only a handful of reports on the plasticity of T cells and their ability to change their phenotype depending on their cytokine milieu and TCR activation by APCs (J. Zhu and Paul 2010).

1.2 Cysteinyl Leukotrienes

1.2.1 Biosynthesis of cysteinyl leukotrienes

Cysteinyl leukotrienes are potent lipid mediators synthesized from arachidonic acid in response to different immune and inflammatory stimuli. They have recognised roles in respiratory diseases such as asthma and allergic rhinitis, but have also been implicated in other inflammatory conditions (Funk 2001; Samuelsson and Hammarstrom 1982).

Although cysteinyl leukotrienes cannot be stored, they can be generated very rapidly at sites of inflammation using *de novo* synthesis (Luster and Tager 2004). Upon cellular activation, phospholipase A_2 (PLA_2) translocates from the cellular cytosol to the nuclear membrane and releases arachidonic acid (AA) from cell-membrane phospholipids (Clark et al. 1990). AA is required for the biosynthesis of leukotrienes and is firstly converted to 5-HPETE by 5-lipoxygenase (5-LO) (see figure 1.4). This 78 kDa monomeric enzyme contains a non-heme iron atom essential for enzyme activity (Percival 1991; Rouzer and Samuelsson 1985). 5-HPETE can be further converted, enzymatically or non-enzymatically, to 5-HETE. Alternatively, 5-HPETE is converted to the unstable intermediate LTA_4 by 5-LO and 5-LO activating protein (FLAP). FLAP is an 18kDa membrane-associated protein that does not have any enzymatic activity but is thought necessary for the interaction of 5-LO with arachidonic acid, by presenting free AA to 5-LO (Abramovitz et al. 1993; Mancini et al. 1993). Leukotriene A_4 can be enzymatically converted to LTB_4 by LTA_4 hydrolase, or LTC_4 by LTC_4 synthase. LTA_4 hydrolase is essential and acts by hydrolysing the epoxide of LTA_4 (Radmark et al. 1984). LTC_4 synthase acts by conjugating a glutathione molecule to LTA_4. Neutrophils and monocytes possess LTA_4 hydrolase and therefore follow the LTB_4 synthesis

pathway; other cell types such as mast cells and eosinophils express LTC_4 synthase and conjugate LTA_4 with the tri-peptide glutathione to form the first of the cysteinyl leukotrienes LTC_4. Both LTB_4 and LTC_4 are then exported from the cell to the surrounding environment, where LTB_4 binds known receptors BLT_1 and BLT_2. Once transported outside of the cell, the gamma glutamyl residue of the glutathione side chain of LTC_4 is cleaved by gamma glutamyl transpeptidase to form LTD_4 (Hammarstrom et al. 1985; Orning and Hammarstrom 1980). And LTE_4 results from the cleavage of the peptide bond between the cysteinyl and glycine residues of LTD_4 by a di-peptidase (C. W. Lee et al. 1983; Lewis et al. 1990). This process can be stopped by L-cysteine. In the body, LTC_4 is rapidly metabolized. The stable LTE_4 is catabolized in the liver predominantly by N-acetylation and excreted in the urine and bile. The concentration of LTE_4 can be measured in urine using sensitive immunoassays and reflects endogenous LTC_4 production (Sansom et al. 1997).

As 5-LO is only found in myeloid cells such as neutrophils, eosinophils, basophils, monocytes/macrophages and mast cells, other non-myeloid cells that lack the required enzyme rely on LTA_4 transferred from surrounding cells, in order to produce leukotrienes. This process is called *transcellular biosynthesis* or *transcellular metabolism* (Luster and Tager 2004; Nicosia et al. 2001), and enables the cysteinyl leukotriene cascade to be initiated. Following release from inflammatory cells, LTA_4 is taken up by nearby cells or platelets, which metabolize it into LTB_4 or LTC_4 (McGee and Fitzpatrick 1986).

Whereas LTB_4 is best known for its role in neutrophil chemotaxis and inflammation, cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 promote recruitment of inflammatory cells

into tissues, such as eosinophils and neutrophils (Krauss et al. 1994; Spada et al. 1994), and cause bronchoconstriction. However, both LTB_4 and cysteinyl leukotrienes amplify inflammation mediated by Th2 cells, particularly allergic responses (Peters-Golden and Henderson 2007).

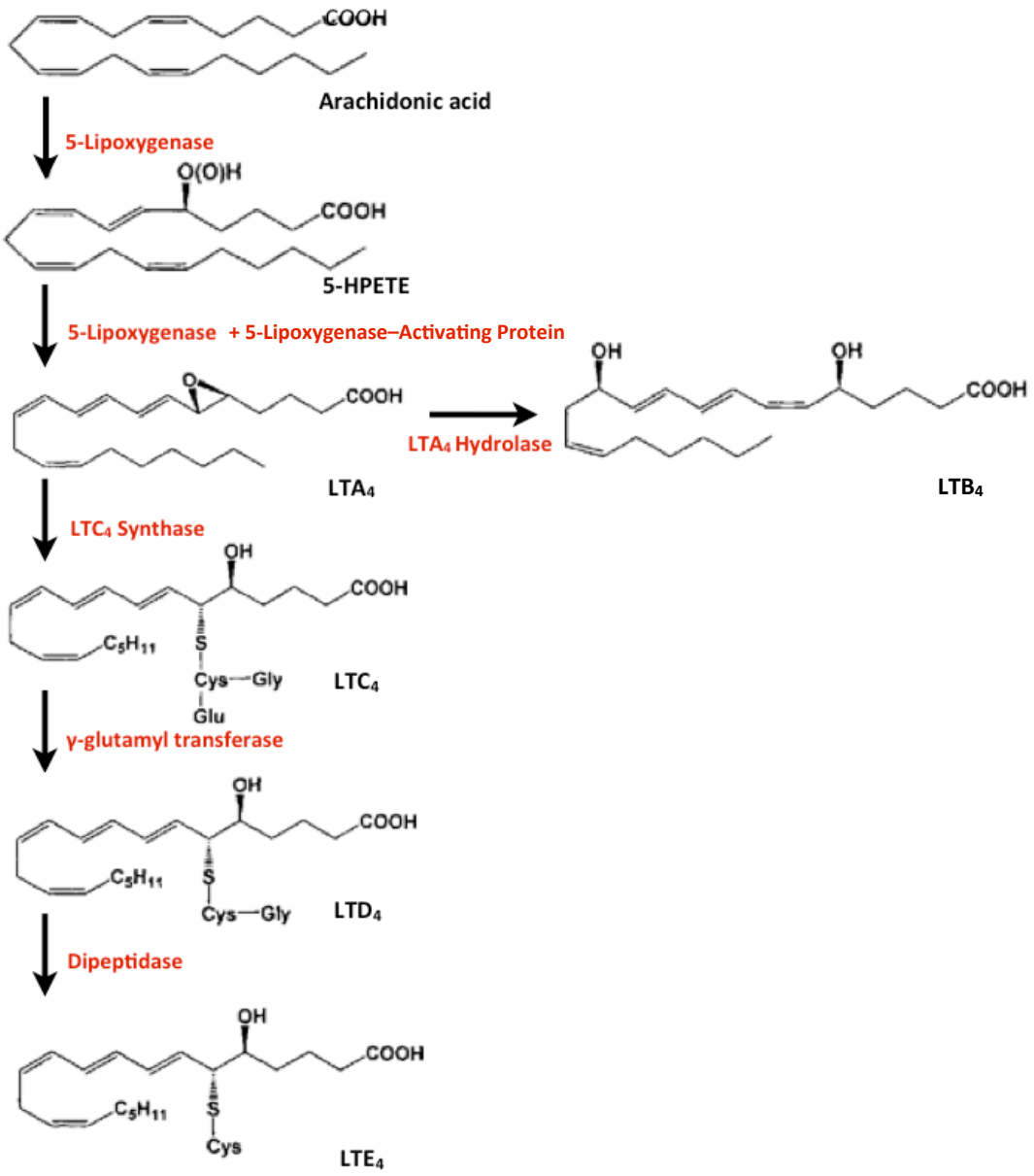


Figure 1.4 Biosynthesis of cysteinyl leukotrienes**.** Arachidonic acid is released from membrane phospholipids with the help of phospholipase A2 and is converted into 5-(*S*)-hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequently into LTA_4 by 5-LO. LTA_4 can be either hydrolyzed into LTB_4 by LTA_4 hydrolase or converted into LTC_4 via conjugation with glutathione by LTC_4 synthase. Removal of the glutamyl group of LTC4 by γ-glutamyl transferase forms LTD_4, which can be cleaved further into LTE_4 by a dipeptidase enzyme (adapted from (Montuschi and Peters-Golden 2010)).

1.2.2 The role of leukotrienes in asthma

Cysteinyl leukotrienes have long been implicated in inflammation and allergic mechanisms, particularly asthma, for being active components of the 'slow-reacting substance of anaphylaxis' (SRS-A). The name SRS-A was given to a substance that was able to contract smooth muscle when antigen-sensitized guinea pig lungs were stimulated (Kellaway CH 1940). The name is derived from the original name SRS (or slow reaction smooth muscle-stimulating substance) given by Feldberg and colleagues (Feldberg 1938) after observations of contraction of an isolated guinea pig ileum by snake venom perfusion.

The evidence for the importance of leukotrienes in asthma stems from two aspects: the pathophysiological responses induced by leukotrienes and the effectiveness of anti-leukotrienes agents. Leukotrienes induce constriction, increase mucus production and vascular leakage and attract eosinophils to the airways (Lewis et al. 1990). When inhaled, LTC_4 and LTD_4 are 1000 times more potent bronchoconstrictors than histamine (N. C. Barnes et al. 1984). Leukotrienes have also been measured in biological fluids of asthmatic patients. Reports have revealed the presence of high concentrations of leukotrienes in sputum from asthmatic individuals, with high levels correlating to the severity of asthma (Pavord et al. 1999). Increased concentrations of leukotrienes in bronchoalveolar lavage fluids of asthmatic patients have also been reported, including those with nocturnal asthma, compared with normal subjects (S. E. Wenzel et al. 1995). Additionally higher levels of LTC_4 and LTD_4 have been found in nasal secretions of patients with symptomatic rhinitis than in those of non-allergic patients (Knani et al. 1992).

The biological relevance of leukotrienes in asthma is further confirmed by the presence of increased levels of LTE_4 in the urine of patients following allergen inhalation (Manning et al. 1990b), exercise challenge (Kikawa et al. 1992), aspirin challenge (Israel et al. 1993a) and asthma exacerbations (Taylor et al. 1989). Studies involving LTE_4 have shown that patients with asthma were 26 times more sensitive to LTE_4 than healthy individuals, compared with 7 times more sensitive to histamine. Additional studies of asthmatics versus healthy individuals revealed that airway hyper-responsiveness was higher by 14-fold in asthmatic patients in response to histamine, 16-fold for methacholine, 6-fold for LTC_4, 9-fold for LTD_4 and 219-fold for LTE_4, suggesting a possible alternative bronchoconstriction mechanism for LTE_4 (Arm et al. 1990). LTE_4 was also the only cysteinyl leukotriene capable of enhancing airway hyper-responsiveness of agonist histamine. Also, a much higher dose of antagonist was needed to inhibit LTE_4-induced increase of response to histamine, in comparison to CysLT-induced contractions (T. H. Lee et al. 1984), suggesting the presence of other receptors by which LTE_4 might operate.

Cysteinyl leukotrienes (CysLTs) play a particularly important role in exercise-induced asthma (EIA), a condition characterised by bronchoconstriction after a period of exercise. Increased levels of CysLTs were found in induced sputum and exhaled breath condensate in asthmatics with EIA. The CysLT1R antagonist montelukast has been demonstrated to be efficacious in the prevention of EIA (Hallstrand and Henderson 2010).

Further supporting evidence for the role of leukotrienes in asthma is anti-leukotriene therapy, which improves the quality of life of asthma patients, such as improved

pulmonary function, decreased daytime/nocturnal symptoms, and reduced need for short-acting β_2-agonists (Britton 1992; Pearlman et al. 1992). CysLT1R antagonists also improve lung function, reduce the rate of exacerbation, and airway and blood eosinophilia (Montuschi and Peters-Golden 2010). Commercially available anti-leukotriene drugs include Zileuton, which inhibits 5-lipoxygenase, and which has been approved for the prevention and chronic treatment of asthma in adults and children of 12 years of age and older in the UK and USA. Others include Montelukast, Zafirlukast, Pranlukast and Pobilukast and block the CysLT1 receptor (Brink et al. 2003). In randomized, double-blind, clinical trials, these drugs are consistently superior to placebo in attenuating airway responses in many clinical models of asthma. For example, MK571 (Manning et al. 1990b) inhibited exercise-induced bronchoconstriction by 50-70%. Zileuton attenuated cold-air induced (Israel et al. 1990) and aspirin-induced (Israel et al. 1993b; Israel et al. 1993a) bronchoconstriction. CysLT1R antagonists provide an improvement in asthma control, although low-dose inhaled corticosteroids remain generally more effective in patients with persistent asthma who remain symptomatic using short-acting β2-agonists alone (Busse et al. 2001). In asthmatic patients who are not sufficiently controlled with a dose of inhaled budesonide alone, add-on therapy with Montelukast improves asthma control to a level that is comparable to the one achieved if the budesonide dose was doubled (Price et al. 2003; Vaquerizo et al. 2003). The role of cysteinyl leukotrienes in allergen-induced bronchoconstriction is clearly demonstrated by the ability of the receptor antagonists to attenuate allergen-induced early and late asthma responses (Hamilton et al. 1998; O'Byrne 1998).

1.3 Leukotriene receptors

1.3.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) form the largest and most diverse superfamily of cell surface receptors and proteins in the mammalian genome (Kroeze et al. 2003), characterised by an extracellular N terminus, an intracellular C terminus and a seven trans-membrane domain. They are also referred to as 7TM receptors. They can be grouped into 6 different classes based on sequence homology and functions: Rhodopsin, Secretin, Adhesion, Glutamate and frizzled receptor families. There are several hundred different GPCRs in the human genome, most of which belong to the rhodopsin family. The primary function of GPCRs is to transduce extracellular stimuli into intracellular signals, which is achieved via interactions between the intracellular domains of the receptor and a heterotrimeric G protein (see Figure 1.5). Receptors that couple to G proteins communicate signals from a large number of external stimuli, which include light, hormones, neurotransmitters, odorants, biogenic amines, lipids, proteins, amino acids, chemokines, and many others (Marinissen and Gutkind 2001).

Upon ligand binding, the receptor associates with the heterotrimeric G protein, which becomes activated by the exchange of GDP for GTP. The G protein then dissociates into its α and βγ subunits. The βγ subunit gives rise to the activation of phospholipase C, leading to the classical leukocyte activation pathway and cleaving phosphatidylinositol (4,5)-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), while IP3 triggers the release of calcium from intracellular stores. Signalling cascades involve mitogen-activated protein kinase (MAPK), which generates responses such as degranulation

and/or chemotaxis. When signalling is over, the α subunit re-associates with the βγ subunits and terminates the signalling process. Depending on the type of G protein to which the receptor is coupled, different downstream signalling pathways can be activated (Kroeze et al. 2003; Marinissen and Gutkind 2001; Neves et al. 2002), which can lead to the regulation of cellular components, and further effects on homeostasis, behaviour, function and development. There are, however, several known Gα, Gβ and Gγ subunits, which all have different functions (Neves et al. 2002).

GPCRs have the ability to control, downregulate or terminate their activity, and therefore attenuate downstream signalling pathways by processes of receptor phosphorylation, desensitization and internalization. Desensitization involves the uncoupling of the receptor from the G protein by the binding of arrestins to phosphorylated receptors (Ferguson 2001), but this can also occur via arrestin-independent pathways (Gray et al. 2003).

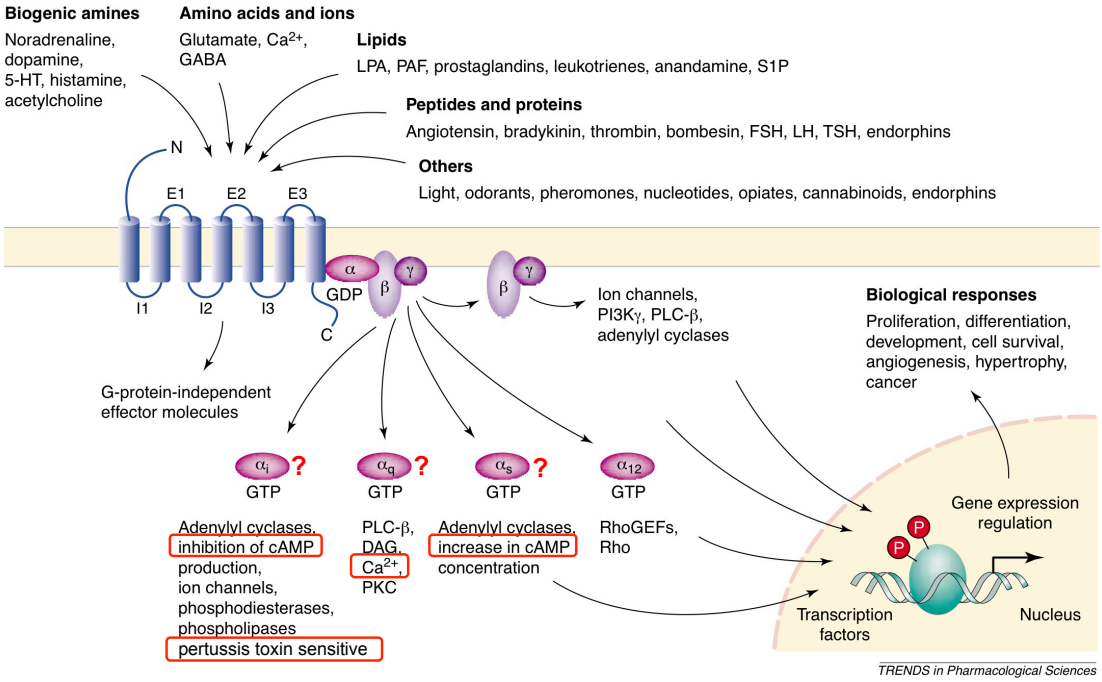


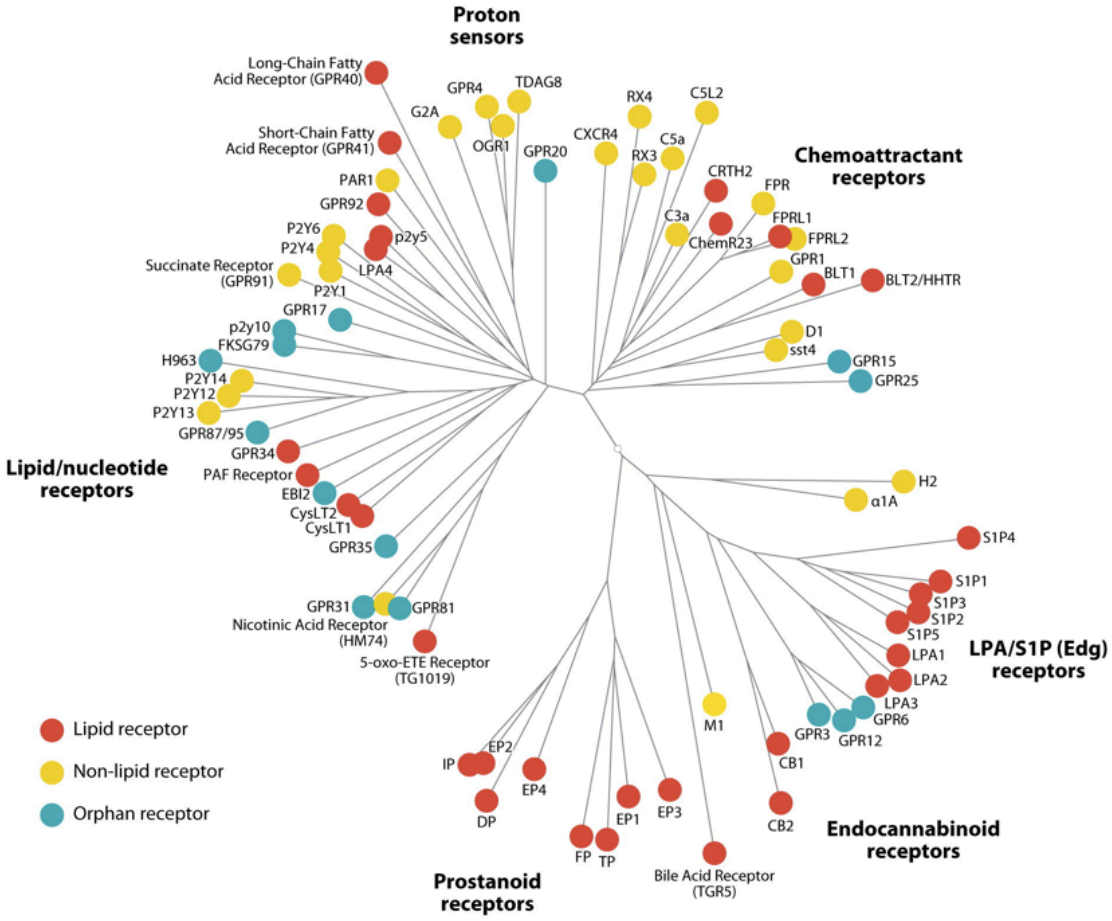
Figure 1.5 Diversity of GPCR signalling. This shows the different signalling pathways and effects of G protein-coupled receptors on downstream functions (Marinissen and Gutkind 2001).

1.3.2 Cysteinyl leukotriene receptors

Leukotrienes act through G protein-coupled seven transmembrane receptors (GPCRs). The four known leukotriene receptors (LT-Rs) belong to two main classes: BLT-Rs (or LTB_4 receptors), which bind LTB_4 and other eicosanoids, and CysLTRs (cysteinyl leukotriene receptors), which bind LTC_4, LTD_4 and LTE_4.

As described earlier, it is thought that LTD_4 activation of the $CysLT_1R$ leads to G protein activation and the release of several second intracellular messengers such as DAG, IP and Ca^{2+}. This is generally followed by the activation of PKC and accompanied by calcium mobilization derived from both intracellular and extracellular calcium stores.

Although stemming from similar molecules, LTB_4 and cysteinyl leukotrienes bind to different receptors. LTB_4 binds known receptors BLT1 and BLT2 (Yokomizo et al. 1997; Yokomizo et al. 2000b; Yokomizo et al. 2000a). On the other hand, cysteinyl leukotrienes bind to the two known receptors $CysLT_1R$ and $CysLT_2R$, which share only 38% homology. Both receptors are seven-transmembrane G protein-coupled receptors and belong to the rhodopsin/β2 agonist family, specifically to the purine receptor cluster of phylogenetically related receptors (see **figure 1.5**). This cluster also includes the receptors for the purinergic or pyrimidinergic nucleotides (P2Ys), proteases (PARs) and PAF (PAFR), in addition to several orphan receptors (Fredriksson et al. 2003; Kroeze et al. 2003).



Shimizu T. 2009.
Annu. Rev. Pharmacol. Toxicol. 49:123–50

Figure 1.6 Phylogenetic tree of G protein coupled receptors and orphan receptors. Taken from (Shimizu 2009).

Both receptors have slightly different binding affinities to the cysteinyl leukotrienes (**see Table 1.1**). $CysLT_1R$ binds LTD_4>LTC_4>>LTE_4, while $CysLT_2R$ binds LTC_4=LTD_4>LTE_4 (Evans 2002). The two receptors also differ in their role: CysLT1R has a more predominant role in bronchoconstriction than $CysLT_2R$, with $CysLT_2$ being involved in inflammation, vascular permeability and tissue fibrosis (Peters-Golden and Henderson 2007).

	$CysLT_1R$	**$CysLT_2R$**
Gene symbol	CYSLTR1	CYSLTR2
Genomic location	Xq13-Xq21	12q14.2
Accession number (GenBank)	AF119711	AB038269
Protein size	337 aa	346 aa
Ligands	$LTD_4 > LTC_4 >> LTE_4$	$LTD_4 = LTC_4 >> LTE_4$
G protein coupling	G_q, G_i, G_o?	G_q, G_i, G_o?
Expression	*High:* Peripheral blood leukocytes, spleen *Low:* placenta, colon, kidney, liver, pancreas, brain, lung	*High:* Heart, adrenal gland, placenta *Low:* Peripheral blood leukocytes, CNS, spleen
Antagonists	MK571, Montelukast, Zafirlukast, Pranlukast, Bay u9773	Bay u9773 (partial agonist)
Induced cytokines	IL-4, IL-13, IFNγ, TGFβ	IL-4, IFNγ

Table 1.1 Comparison between $CysLT_1$ and $CysLT_2$ receptors. Adapted from Rovati and Capra, 2007 (Rovati and Capra 2007).

1.3.3 Cysteinyl leukotriene receptor 1

1.3.3.1 Cloning of CYSLTR1

The human cysteinyl leukotriene receptor 1 or hCysLT$_1$R was first cloned and characterised in 1999 by two independent groups (Lynch et al. 1999; Sarau et al. 1999). As part of a programme to identify ligands for orphan GPCRs, Lynch *et al* identified an expressed sequence tag entry, re-named HG55 at the time, and now known as CysLTR1, that had 32% amino acid homology to the purinoreceptor P2Y1 and PAF and 28% homology to the LTB4 receptor (BLT) which had been cloned the previous year (Lynch et al. 1999). HG55, or the human CysLTR1, was identified as a 337 amino acid putative seven trans-membrane domain receptor with a calculated molecular mass varying between 38kDa and 42kDa as a monomeric form, but usually found in dimeric or oligomeric forms. The human CysLT1 receptor was located to the X chromosome Xq13-Xq21 and is composed of at least 3 exons (Evans 2002). It has 31% amino acid identity to the P2Y purinoceptor (Sarau et al. 1999).

Using two different *Xenopus laevis* functional systems, Lynch *et al* characterised CysLT1R (or HG55) activation. They found that LTD_4 induced a robust dose-dependent calcium response in oocytes injected with CysLTR1 cRNA, and that this response was attenuated with continuous administration of agonist. Oocytes injected with saline or another orphan GPCR cRNA did not respond to LTD_4. When pre-treated with pertussis toxin the result showed a slight inhibition and a much lower response, suggesting a partial signalling via the Gαi pathway in oocytes. When treated with the known CysLTR1 selective antagonist MK-571, the calcium responses to LTD_4 were inhibited in oocytes in a dose-dependent manner. Similar results were achieved when the group

added LTC_4, LTD_4, and LTE_4 to *Xenopus laevis* melanophores transfected with CysLTR1 cDNA and found that all three cysteinyl leukotrienes caused activation of pigment dispersion, in a dose-dependent manner. In this system, LTD_4 was the most potent agonist, while LTC_4 was slightly weaker and LTE4 very weak. When transfected melanophores were pre-treated with antagonist MK-571, the response was blocked. Similar results were found in mammalian monkey kidney COS-7 cells expressing the human CYSLTR1 (Lynch et al. 1999). Using aequorin luminescence and calcium green – 1 fluorescence, the group measured the intracellular calcium mobilization in response to LTD_4 in COS-7 monkey kidney cells transiently expressing CYSLTR1. They found that all three LTs caused a calcium response in the COS-7 cells, in contrast to control cells which did not respond. Again they found that there was a rank order of potency with LTD4 being most potent (LTD_4>LTC_4>LTE_4). As in previous experiments, MK571 blocked the response. Saturation analysis studies of [^{3}H]LTD_4 binding to membranes revealed an order of affinity of $LTD_4 \geq LTE_4 = LTC_4 \geq LTB_4$. $CysLT_1$ receptor antagonists MK571, montelukast (Singulair), zafirlukast (Accolate) and pranlukast (Onon) all showed high affinity for the $CysLT_1$ receptor. Northern blot analysis studies of CYSLT1R mRNA in various human tissues indicated the highest expression in the spleen and peripheral blood leukocytes, and a lower expression in various tissues including the lung. Given the important role of $CysLT_1R$ in asthma, the group decided to investigate CYSLT1R mRNA distribution in the lung using *in situ* hybridization, using an oligonucleotide probe, and found it to be present in peribronchial smooth muscle cells, including segmental bronchi and bronchioles, in smooth muscle bundles and in lung macrophages (Lynch et al. 1999).

At the same time, Sarau *et al.* also investigated the molecular cloning, expression, localization, and pharmacological characterization of the $CysLT_1$ receptor (Sarau et al. 1999). Using public and private ESTs (Expression Sequence Tags) converted into full-length cDNAs and transiently or stably expressed in human embryonic kidney (HEK293) cells, and using the FLIPR (Fluorescence Imaging Plate Reader) method, the group identified a transiently transfected receptor cell line that responded specifically to LTC_4 and LTD_4. The FLIPR Assay method uses a calcium sensitive dye that is taken into the cytoplasm of the cell during incubation. The particular masking technology remains outside the cell and blocks background fluorescence. Upon ligand binding to the receptor, calcium is released into the cytoplasm of the cell. The dye binds to the intracellular calcium and becomes fluorescent. Sequence analysis revealed the full length of this cDNA sequence was 1579 bp and encoded a protein of 337 amino acids, which they identified as $CysLT_1R$. The group found that in mammalian HEK293 cells transfected with $CysLT_1R$, the human CysLTR1 responded selectively to cysteinyl leukotrienes LTD_4>LTC_4>LTE_4 in that rank order of potency, and with EC_{50} values of 2.5 nM, 24 nM and 240 nM, respectively. Of 900 ligands tested, the group found that, aside from cysteinyl leukotrienes, none elicited a specific calcium response in HEK293-$CysLT_1R$ transfectants. They tested the LTD_4 response in COS-7- and CHO- transfected cells and found a strong calcium response similar to Lynch *et al*. All calcium responses were inhibited calcium mobilization induced by LTD_4 in those cells was inhibited by the structurally distinct $CysLT_1$ receptor antagonists pranlukast, zafirlukast, montelukast and pobilukast. The rank order of potency for these antagonists was pranklukast=zafirlukast>montelukast>pobilukast (Sarau et al. 1999). Interestingly, pre-treatment with pertussis toxin did not affect the calcium response and the calcium

response was still observed after removal of extracellular calcium. Radio-ligand binding studies confirmed the affinity results observed by Lynch *et al.* Similarly, northern blot analysis studies of human tissues revealed localization and a high expression of human CYSLTR1 mRNA in the spleen and peripheral blood leukocytes, less so in the lung, small intestine, pancreas and placenta, with little or no expression in the liver, colon, kidney, skeletal muscle, thymus, ovary, testis, heart and brain (Lynch et al. 1999).

Human CYSLTR1 has been reported to be expressed by a variety of airway mucosal inflammatory cells involved in asthma, including bronchial mucosal eosinophils, neutrophils, mast cells, macrophages, B lymphocytes and plasma cells. Interestingly, CYSLTR1 expression has not been reported in T lymphocytes (Figueroa et al. 2001).

Two isoforms of the mouse $CysLT_1$ receptor have been cloned (Maekawa et al. 2001; V. Martin et al. 2001; Mollerup et al. 2001): a short and a long isoform. The short isoform cDNA contains 2 exons and encodes a polypeptide of 339 amino acids with 87.3% amino acid identity to the human $CysLT_1R$. The long isoform has 2 additional exons and an in-frame upstream start codon, which results in a 13-amino acid extension at the N-terminus (Lynch et al. 1999; Maekawa et al. 2001; Sarau et al. 1999). The long isoform resembles the human receptor the most and is found more abundantly in the mouse tissues. The mouse Cysltr1 is found on chromosome X at band XD. Like in the human system, LTD_4-induced calcium mobilisation was also blocked by MK571. The receptor binding affinities to cysteinyl leukotrienes were similar (Lynch et al. 1999; Maekawa et al. 2001; Sarau et al. 1999).

1.3.3.2 Cellular and tissue distribution

The expression of CYSLTR1 has been extensively studied since the first cloning reports in 1999. Figueroa *et al.* have examined the expression of CYSLTR1 in normal human lung tissue and peripheral blood leukocytes. Using immunohistochemistry, they investigated the distribution of the receptor protein in smooth muscle cells of normal human lung and in macrophages, as well as peripheral blood leukocytes. Using a specific oligonucleotide probe (antisense), the group identified CYSLTR1 mRNA and protein expression in smooth muscle fibres. While the antisense probe had already previously been used to recognise CYSLTR1 mRNA in human lung muscle cells and macrophages (Lynch et al. 1999), this was the first report using antisera designed to recognise CysLTR1. The antisera were raised in goat and recognised purified CysLTR1 specifically on immunoblot analysis. However they were unable to detect any receptor in immunoblot analyses of lung tissue or COS cells transfected with CysLTR1 membrane fractions, which they suggested was due to the receptor levels being too low to detect. They suggested that much larger amounts of dimerized and oligomerized $CysLT_1$ receptors were observed than the monomeric recombinant CysLTR1 of molecular weight 42kD, however the blots were not very clear. However using immunohistochemistry on COS cells transfected with $CysLT_1R$, they found positive staining for CysLTR1 using the antiserum, in contrast to COS cells alone. Using the specific antiserum, the group found CysLTR1 protein expression in airway smooth muscle cells. Further experiments using PBMCs from which T cells had been removed revealed similar CYSLTR1 mRNA and protein expression in 20% of the total cell population, and positive cells were also found to express CD14 monocytic and CD19 B-lymphocytic cells markers. CYSLTR1 expression was also observed in CD34+

granulocytic precursor cells, cells expressing the IL-5β receptor, eosinophils from non-asthmatic individuals and in human lung interstitial macrophages. Interestingly, the data showed no evidence of the CYSLTR1 mRNA or protein expression in purified T cells isolated from PBMCs from non-asthmatic subjects. Neither CYSLTR1 mRNA or protein expression was observed in either CD4+ or CD8+ helper T cells (Figueroa et al. 2001).

Zhu *et al.* used *in situ* hybridization and immunohistochemistry in order to identify and quantify inflammatory cells expressing CYSLTR1 mRNA and protein on endobronchial biopsy tissue (J. Zhu et al. 2005). The studies to identify CYSLTR1 mRNA and protein expression involved using 3 subjects groups: non-smoking control subjects with normal lung function, stable asthmatics (non-smoking with mild persistent asthma) and severe asthmatics. The group used IHC and observed positive staining for CysLTR1 protein in CysLTR1 cDNA- transfected COS cells, using a selective, affinity purified, anti-human polyclonal antisera for $CysLT_1$ receptor. The group then used double immunohistochemistry to identify the specific immunophenotypes of the cells in biopsy samples. They identified CYSLTR1 mRNA and protein in inflammatory cells from the subepithelium, which were more abundant in stable and severe asthmatics than in control subjects. Some of the cells that stained positive were also found to infiltrate the surface epithelium. CYSLTR1 mRNA and protein were found in vascular endothelium from all subjects. Using double immunostaining they identified CysLT1R receptor protein co-expression in bronchial inflammatory cells, including eosinophils, neutrophils, mast cells, macrophages, B-lymphocytes, plasma cells and monocytes. Furthermore, expression was higher in asthmatics subjects, with significantly higher

numbers in severe asthmatics. Interestingly, they found no expression of the $CysLT_1$ receptor in bronchial CD3+ T cells, CD4+ T cells and CD8+ T cells. Strong variability was seen across all subject groups for CYSLTR1 mRNA and protein in airway smooth muscle myocytes. Quantification analyses revealed a strong positive correlation between numbers of CD45+ leukocytes and the number of cells expressing $CysLT_1$ receptor (J. Zhu et al. 2005).

Further studies on human peripheral blood cells have demonstrated expression of CysLT1R on various cell types.

Thivierge *et al.* showed that CYSLTR1 mRNA was constitutively expressed by human monocytes and treatment with IL-13 augmented the CYSLTR1 mRNA levels by 3- to 5-fold. This increase was time-dependent and observed as early as 4 hours post stimulation, with a maximal response at 8 hours. This effect was maintained for 24 hours. The group also showed that they obtained similar results by treating the cells with IL-4, but not IFN-y. In both cases, protein levels were also increased (Thivierge et al. 2001).

Studies on human platelets have also revealed expression of CYSLTR1. CYSLTR1 mRNA was identified in human platelets from healthy individuals. Using an anti-$CysLT_1R$ antibody, $CysLT_1R$ expression was found by flow cytometry on the surface of human platelets and positive control cells. Protein expression was also detected in human platelets by western blotting (Hasegawa et al. 2010).

CYSLTR1 mRNA was also identified in chronic lymphocytic leukemia (CLL) cell lines MEC-1 and EHEB, and in $CD19^+$ B cells from CLL patients. Additional calcium studies

showed that LTD_4 induced calcium flux in both MEC-1 and EHED cells lines and this was measured using fluorescent Ca^{2+} indicator fluo-3 AM and flow cytometry. Calcium mobilisation occurred via G protein Gαi, as indicated by experiments using pertussis toxin. All calcium flux was inhibited when using CysLT1R antagonists MK571 and LY171883 (Drost et al. 2012).

Woszczek *et al*. investigated steady-state levels of mRNA for 2 known CysLT receptors in human monocytes and found that CYSLTR1 mRNA was the most highly expressed, followed by 16-fold lower levels of CYSLTR2 mRNA. Interestingly they could not detect GPR17 mRNA (Woszczek et al. 2008b). The group also stimulated monocytes with different concentrations of LTC_4, LTD_4 and LTE_4 to find that all three of them induced intracellular calcium mobilization, with LTD_4 showing the highest potency, followed by LTC_4 and LTE_4. Pre-treatment of human monocytes with MK571 inhibited the calcium response, as did pre-treatment with pertussis toxin, 2APB, and U73122, suggesting that calcium flux was induced by $CysLT_1$ receptor coupled to Gαi, followed by activation of phospholipase C and IP3 signaling. Pre-incubation with the extracellular calcium-chelating agent EGTA and with the intracellular calcium-chelating agent BAPTA/AM inhibited calcium mobilization, suggesting both intracellular and extracellular calcium are required. Pre-incubation with thapsigargin to deplete endoplasmic reticular calcium stores blocked the previously observed calcium response. However pre-treatment with plasma membrane channels inhibitor (L-type) verapamil did not have any effect. These data suggested that calcium flux in human monocytes required store-operated calcium-channel activation by LTD_4 stimulation (Woszczek et al. 2008b).

In a separate study, the same group investigated whether the IL-4-increased CYSLTR1 promoter activity observed previously in gene reporter experiments was functional at mRNA and protein levels. Using RT-PCR and flow cytometry, they found that IL-4 stimulation of THP-1 (acute monocytic leukemia) cells increased CYSLTR1 mRNA expression in a time-dependent manner, with the highest increase observed after 6 h of incubation, as well as a significant time- and dose-dependent increase in surface receptor expression, with the maximum response observed after 24 h of incubation (Woszczek et al. 2005). Culture of human monocytes in the presence of IL-10 was found to decrease CYSLTR1 mRNA expression in a concentration-dependent manner, with the maximum effect observed after 6 hours. IL-10 was found to also decrease CYSLTR1 gene transcription and protein expression in a time-dependent manner (Woszczek et al. 2008a).

1.3.3.3 Murine studies

Studies in mouse models have also reported the expression of Cysltr1 in murine cells and calcium flux caused in response to LTD_4. The following section describes some of the results observed in these studies.

Martin *et al.* first cloned the murine $CysLT_1$ receptor in 2001, and found 87% identity at the amino acid level between the mouse and human receptor. Binding experiments revealed similar results to the human studies and all known specific antagonists competed for binding sites in mouse $CysLT_1R$ ($mCysLT_1R$). Calcium flux was observed in response to LTD_4 in COS-7 cells transfected with $mCysLT_1R$. Calcium flux in HEK293 cells transfected with $mCysLT_1R$ was LTD_4-dose-dependent. Finally, the group also tested *X. laevis* melanophores and transfected them with $mCysLT_1R$. LTD_4 stimulation of

mCysLT$_1$R caused pigment dispersion in a concentration-dependent manner with an EC_{50} value of 0.2 nM (V. Martin et al. 2001).

Several studies have involved Cysltr1$^{-/-}$, Cysltr2$^{-/-}$ knockouts or Cysltr1/Cysltr2$^{-/-}$ double knockout mice. Maekawa *et al.* generated Cysltr1-deficient mice by targeted gene disruption and mice were found to develop normally and to be fertile. Targeted gene disruption was generated by homologous recombination using a targeting vector designed so that the *neo* gene insertion interrupts the coding region that is common to the long and short isoforms of the mouse Cysltr1 gene. Using an intracellular mobilization assay with fura-2 acetoxymethyl ester, the group found that macrophages from Cysltr1-deficient mice did not respond to either LTD_4 or LTC_4, compared to peritoneal macrophages from wild-type littermates (which expressed both Cysltr1 and Cysltr2), which responded to LTD_4 and slightly to LTC_4. They also found that plasma protein extravasation, but not neutrophil infiltration, was significantly reduced in cysltr1-deficient mice subjected to zymosan A-induced peritoneal inflammation. Plasma protein extravasation was also significantly reduced in cysltr1-deficient mice undergoing IgE-mediated passive cutaneous anaphylaxis, in comparison to the wild-type mice (Maekawa et al. 2002)

Later, the same group generated Cysltr2-deficient mice again using targeted gene disruption (as described earlier). These mice developed normally and were fertile, like in previous studies in cysltr1-deficient mice. Importantly, the knockout did not induce any change in the CysLTR1 mRNA expression. They found that in Cysltr2$^{-/-}$ mice, the increased vascular permeability associated with IgE-dependent passive cutaneous anaphylaxis was significantly reduced, in comparison to wild-type mice. Plasma protein

extravasation in response to zymosan A-induced peritoneal inflammation was not altered. To define a role for the $CysLT_2$ receptor in the pathobiology of chronic inflammation with fibrosis, they injected bleomycin into the tracheas of Cysltr2-deficient mice and their wild-type littermates and observed that the lung tissue of bleomycin-treated Cysltr2-deficient mice had less septal thickening, with less accumulation of monocyte/macrophages, giant cells, fibroblasts, and eosinophils and less extracellular matrix deposition than that of the bleomycin-treated wild-type littermates. This suggested that $CysLT_2$ receptor can mediate an increase in vascular permeability in some tissues or promote chronic pulmonary inflammation with fibrosis, in response to a particular pathobiologic event (Beller et al. 2004). Interestingly, the group found that neither the $Cysltr1^{-/-}$ nor the $Cysltr2^{-/-}$ mice were protected from antigen-induced pulmonary inflammation, suggesting that additional receptor(s) might account for the protection observed in previous studies in $Ltc4s^{-/-}$ mice.

Jiang *et al.* reported that LTD_4-mediated proliferation was abrogated in human mast cells (hMCs) following short-hairpin RNA (shRNA)–mediated knockdown of $CysLT_1$ receptor expression, and in mouse bone marrow–derived MCs (mBMMCs) from $Cysltr1^{-/-}$ mice. In contrast, the study showed that knockdown of $CysLT_2$ receptor led to an increase in CysLT1 receptor surface expression and CysLT1-dependent proliferation of cord blood–derived human MCs (hMCs). Although CysLT–mediated responses were absent in MCs from mice lacking $CysLT_1$ receptors, they were enhanced by the absence of $CysLT_2$ receptors. Although the absence of CysLT2 receptor did not affect the total cellular level of CysLTR1 protein, it increased CysLT1 expression at the cell surface and

enhances LTD4-induced ERK phosphorylation. CysLT1 and CysLT2 receptors were found to colocalize at the plasma membrane, nucleus, and nuclear envelope of MCs, and form heteromeric complexes, which was determined by Ab-based fluorescent lifetime imaging microscopy (FLIM) to measure fluorescent resonance energy transfer (FRET) (Jiang et al. 2007).

Following previous findings of a greater potency for LTE_4 than LTC_4 or LTD_4 in constricting guinea pig trachea *in vitro* and comparable activity in eliciting a cutaneous wheal and flare response in humans; and following on from their findings in previous models of Cysltr1$^{-/-}$ and Cysltr2$^{-/-}$ knockout mice, Maekawa *et al.* generated cysltr1/Cysltr2 double-deficient (Cysltr1/Cysltr2$^{-/-}$) mice in order to check for any vascular permeability response to LTE_4 (Maekawa et al. 2008). They examined the agonist function of the three cysteinyl leukotrienes by intradermal injection into the ear and found that the Cysltr1/Cysltr2$^{-/-}$ mice expressed a previously unrecognized receptor that mediated a greater vascular leak in response to LTE_4 than to LTD_4 or LTC_4, which was inhibited by pre-treatment of the mice with pertussis toxin or a Rho kinase inhibitor. The increased sensitivity of the Cysltr1/Cysltr2$^{-/-}$ mice compared with WT mice was approximately 64-fold by dose-dependent analysis, and revealed a negative regulation of the novel unknown receptor for LTE_4 by the two known receptors, with a preference for LTD_4 and LTC_4. Additionally, they found that pre-treatment of Cysltr1/Cysltr2$^{-/-}$ mice with antagonist MK-571 further increased the permeability response of these mice to each of the three cysteinyl leukotrienes (Maekawa et al. 2008).

1.3.4 Cysteinyl leukotriene receptor 2

The human $CysLT_2$ receptor was initially cloned and characterised in 2000 by Heise *et al.* (Heise et al. 2000), followed closely by two other groups (Nothacker et al. 2000; Takasaki et al. 2000). The mouse cysltr2 was also identified in 2001 (Hui et al. 2001). Although from the same family, human CYSLTR2 only shares 37.5% amino acid identity with human CYSLTR1 (Heise et al. 2000). The receptors also have slightly different binding affinities to the cysteinyl leukotrienes. $CysLT_1R$ binds $LTD_4>LTC_4>>LTE_4$, while $CysLT_2R$ binds $LTC_4=LTD_4>LTE_4$ (Evans 2002). Human CYSLTR2 is localised in the 13q14.2 region of chromosome 13. The open reading frame of human CYSLTR2 encodes a protein of 347 amino acids. Northern blot analyses of human CYSLTR2 revealed high expression in the human heart, adrenals, peripheral blood leukocytes, airway smooth muscle, alveolar macrophages, and lower expression in brain cells (Heise et al. 2000). The $CysLT_2$ receptor is also expressed in human peripheral basophils (Gauvreau et al. 2005), endothelial cells (Di Gennaro et al. 2004), cultured mast cells (Mellor et al. 2003), and in nasal eosinophils and mast cells from patients with active seasonal rhinitis (Figueroa et al. 2001).

Human CYSLTR2 cRNA was expressed in *Xenopus laevis* oocytes and both LTC_4 and LTD_4 caused a calcium-dependent chloride flux (Heise et al. 2000). This could not be blocked by selective antagonist MK571, however was inhibited by dual antagonist BAY u9773 (Heise et al. 2000). In HEK 293 cells, LTC_4- and LTD_4-induced calcium mobilization was concentration-dependent in cells expressing the recombinant human CYSLTR2 and was blocked by BAY u9773 but not significantly by $CysLT_1$ receptor antagonists MK571, montelukast, zafirlukast, or pranklukast (Heise et al. 2000). To date a selective human $CysLT_2$ receptor antagonist remains to be identified.

1.3.5 The third receptor

Over the years, several data in the literature have suggested the existence of an additional CysLTR subtype in human tissues. This proposal has been based on several findings and observations. Certain reported actions of cysteinyl leukotrienes cannot be explained by their ligation to either $CysLT_1R$ or $CysLT_2R$. The bronchoconstrictor activity of LTE_4 that has been observed by several studies cannot be explained by either $CysLT_1R$ or $CysLT_2R$ given its low affinity to both (Davidson et al. 1987).

Most importantly LTE_4, the most stable CysLT, is only a weak agonist for the defined $CysLT_1R$ and $CysLT_2R$, despite its greater potency in constricting guinea pig trachea *in vitro* and comparable activity in eliciting a cutaneous wheal and flare response in humans. With this in mind, Maekawa *et al*. hypothesized that a vascular permeability response to LTE_4 in mice lacking both the Cysltr1 and Cysltr2 could establish the existence of a separate LTE_4 receptor. Using double knockout mice deficient in both Cysltr1 and Cysltr2, they administered an intradermal injection of LTE_4 into the ear of the mice. This elicited a vascular leak that exceeded the response to intradermal injection of LTC_4 or LTD_4. Pre-treatment of the mice with pertussis toxin or a Rho kinase inhibitor inhibited the response. Interestingly, it was found that LTE_4 was 64-fold more potent in the Cysltr1/Cysltr2 double-deficient mice than in genetically sufficient mice (Maekawa et al. 2008).

These studies suggest and provide evidence for the existence of one or more unknown additional receptors, including one specific to LTE_4. So far two candidates have been proposed: GPR17 and $P2Y_{12}$.

Recently, the orphan G protein-coupled receptor GPR17 has been identified as a third putative cysteinyl leukotriene receptor capable of binding LTC_4 and LTD_4 (Ciana et al.

2006). GPR17 has been characterized as a dual receptor for uracil nucleotides and cysteinyl leukotrienes. Its expression has been detected in the brain, kidney, heart and human umbilical cord endothelial cells (Ciana et al. 2006) using semi-quantitative PCR. Although its biological role still remains unclear, studies using transfected cells have shown that GPR17 responds to cysteinyl leukotrienes in the same concentration range as $CysLT_1R$ and $CysLT_2R$, however with slightly different ligand binding affinity: LTC4>>LTD4. Montelukast and Pranlukast were both found to block the receptor. GPR17 was also found to negatively regulate $CysLT_1R$-mediated allergic responses in a mouse model of asthma (Maekawa et al. 2010).

$P2Y_{12}$, a target of clinically well-accepted anti-platelet thienopyridine derivatives such as clopidogrel and ticlopidine, has been recently proposed as the receptor for LTE_4. In studies using P2Y12 knockout mice, P2Y12 antagonist clopidogrel was found to suppress *in vivo* enhancement of eosinophilic lung inflammation by LTE_4 inhalation, but not in Cysltr1/Cysltr2 double knockout mice (Paruchuri et al. 2009). However, the group was unable to confirm direct binding of LTE_4 to $P2Y_{12}$.

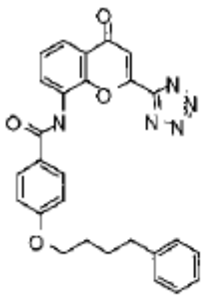
1.3.6 Cysteinyl leukotriene receptor antagonists

Currently, four selective $CysLT_1$ receptor antagonists are clinically available, Montelukast, Zafirlukast, Pranlukast and Pobilukast, with Montelukast being the most widely available and actively marketed (Okunishi and Peters-Golden 2011). As these all selectively antagonize the $CysLT_1$ receptor subtype and all have very similar *in vitro* and *in vivo* preclinical profiles, they mainly differ in terms of relative potency and oral bioavailability (Aharony 1998). The potency and oral activity of Montelukast are similar to those of Zafirlukast, whereas Pobilukast is less potent in binding to the human

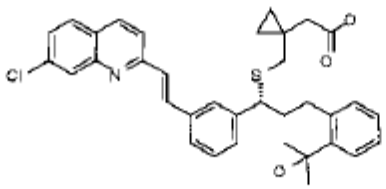
receptor, less effective orally and is delivered by aerosol to achieve effective antagonism (Aharony 1998). Pranlukast also appears to be less potent but is effective when administered orally (Aharony 1998). Although clearly superior to placebo controls, these drugs have limited efficacy, which include their inferiority to ICS in anti-inflammatory and clinical effects and their non-responsiveness on a considerable subset of patients, approximately 50% (P. J. Barnes and Stockley 2005; Terashima et al. 2002). Hypotheses for their limitations include that the asthmatic patient may not produce or produce enough CysLTs for them to become therapeutic targets, but also that anti-leukotrienes may target $CysLT_1$ or $CysLT_2$ receptors instead of a potential additional receptor. Other leukotriene antagonists include selective $CysLT_1$ receptor antagonists Pobilukast and ICI 198615, and non-selective antagonists FPL 55712 and dual receptors antagonist BAY u9773 (Brink et al. 2003).

However these drugs have also been shown to be useful for certain clinical situations. Indeed, LTRAs (leukotriene receptor antagonists) are clinically efficacious in patients with aspirin-induced asthma and exercise-induced asthma (EIA). In EIA patients who show an increase in airway cysteinyl leukotrienes levels following exercise, LTRAs have been found to be superior to ICS in children and superior to long-acting beta agonists in adults. Anti-leukotriene agents also have the additive benefit in patients whose disease cannot be adequately controlled by inhaled corticosteroids. Additionally, anti-leukotriene agents can be taken orally, instead of inhalation (Brink et al. 2003).

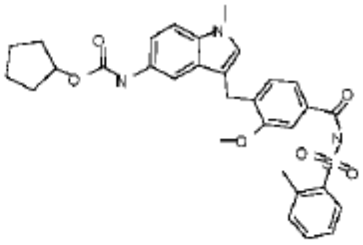
$CysLT_1$ selective antagonists



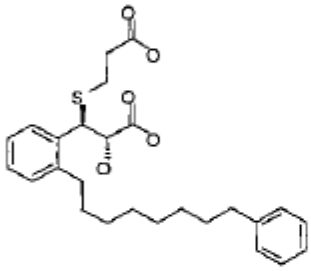
Pranlukast
(Ultair)



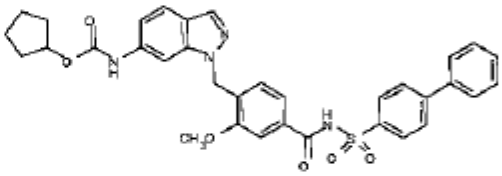
Montelukast
(Singulair)



Zafirlukast
(Accolate)

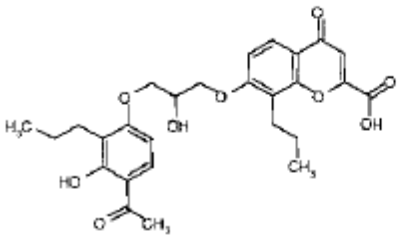


Pobilukast

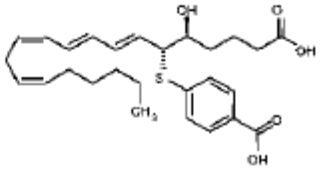


ICI 198615

Non-selective antagonists



FPL 55712



BAY u9773
($CysLT_1$/$CysLT_2$ dual antagonist)

Figure 1.7 Chemical structures of selective and non-selective $CysLT_1$ receptor antagonists (Taken from (Brink et al. 2003).

1.3.7 Functional studies on cysteinyl leukotrienes

Several functional studies on cysteinyl leukotrienes support the hypothesis that they might serve as chemotactic mediators and/or activating ligands for human effector leukocytes. Studies have also shown that some cytokines and other environmental factors in inflammation may affect the CYSLTR1 receptor expression profile in certain leukocytes (Kanaoka and Boyce 2004).

In addition to bronchoconstriction, inhalation of LTE_4 by asthmatic patients has been shown to result in the subsequent influx of eosinophils and neutrophils into the BAL fluid (Laitinen et al. 1993), suggesting a role for cysteinyl leukotrienes in direct or indirect recruitment of leukocytes to sites of inflammation.

In a study using human CD34+ peripheral blood-derived progenitor cells, they found the expression of CYSLTR1 but not CYSLTR2 on these cells. Cysteinyl leukotrienes induced transendothelial migration of these cells in vitro, suggesting a role in the regulation of cell trafficking. Pre-treatment of these cells with antagonist Montelukast completely blocked CysLT-mediated calcium flux (Bautz et al. 2001).

Additional studies in peripheral blood monocytes, eosinophils and lung macrophages all revealed expression of both CYSLTR1 and CYSLTR2 mRNA and protein (Figueroa et al. 2001; Lynch et al. 1999; Mita et al. 2001).

Thivierge *et al*. also showed that priming of either human peripheral blood monocytes or monocyte-derived macrophages with IL-13 or IL-4 increased their levels of CYSLTR1 mRNA and protein expression, as well as enhanced their chemotactic response to LTD_4 *in vitro* (Thivierge et al. 2001).

Cysteinyl leukotrienes have also been observed to induce cytokine generation by eosinophils and mast cells. Human placental cord eosinophils have been found to secrete IL-4 in response to cysteinyl leukotrienes and via $CysLT_1R$. However this was not found in freshly isolated peripheral blood eosinophils unless cells were permeabilized. The expression of IL-4 by permeabilized eosinophils was shown to be PTX-sensitive and was stronger using smaller doses of LTC_4 than LTD_4. Interestingly, pre-treatment of eosinophils with MK886 (FLAP and LTC4S inhibitor) or AA861 (5-LO inhibitor) blocked the release of IL-4 in response to IL-16, RANTES or eotaxin, which suggests an intracrine role for cysteinyl leukotrienes (Bandeira-Melo and Weller 2003; Figueroa et al. 2003).

Expression of both CYSLTR1 and CYSLTR2 has been identified in resident tissue mast cells from nasal biopsies and human cord blood. Mast cells have been shown to respond to ex vivo stimulation of low concentrations of LTC_4 and LTD_4 by strong calcium flux. Calcium flux was pertussis toxin-sensitive and inhibited by $CysLT_1$ receptor antagonist MK571. When primed with IL-4, human mast cells responded better to LTC_4 than LTD_4, as observed by the greater extent of the LTC_4 dose-response curve. However the IL-4 priming did not alter the CYSLTR1 mRNA or protein expression levels. Similar calcium results were observed using UDP, which were also inhibited by MK571. Mast cells were also found to secrete cytokines IL-5, TNF-α, MIP-1β and IL-8 when stimulated with LTC4, LTD4 or UDP. Interestingly, of all cytokines produced, IL-8 was the only one resistant to MK571. Interestingly, it was suggested that the induction of IL-5 by mast cells could be an additional mechanism by which cysteinyl leukotrienes could promote eosinophilia (Mellor et al. 2001; Mellor et al. 2002; Mellor et al. 2003).

The role of cysteinyl leukotrienes and their receptors in immune responses has also been assessed in mice. In a study by Henderson, BALB/c mice were sensitized with an i.p. injection of OVA precipitated with alum then challenged with intranasal administration of OVA. Levels of LTC_4 and LTB_4 were measured in the BAL fluid 24 hours after challenge and found to have increased 5-fold and 3.4-fold, respectively, compared to saline controls. Increased levels of LTC_4 and LTB_4 were associated with widespread mucus obstruction of the airways, an influx of eosinophils in airway tissues and BAL fluid, and airway hyperresponsiveness to intravenous infusions of methacholine. Administration of inhibitors Zileuton or MK886 before nasal antigen challenge reduced eosinophil infiltration in tissues and BAL fluid by approximately 85% and significantly blocked airway mucus release (Henderson et al. 1996). The group reported a similar study but with a period of intranasal administration of OVA of 75 days this time in order to induce the known features of airway remodelling such as smooth muscle hyperplasia and sub-epithelial fibrosis. Results showed that treatment with montelukast on day 26 significantly reduced the infiltration of eosinophils in the airways, mucus obstruction, collagen deposition, and smooth muscle hyperplasia. Compared to placebo-treated challenged controls, montelukast-treated mice showed reduced levels of IL-13, IL-4, IL-10 an IL-5 mRNA, which suggested that cysteinyl leukotrienes may play a role in stimulating cytokine generation by resident inflammatory cells (Henderson et al. 2002).

1.4 Rationale and aim

Asthma has been described as a Th2-specific inflammatory disorder, characterised by symptoms of wheezing and breathing difficulties, and at the cellular level by mucus production, airway hyperresponsiveness and bronchoconstriction. Cysteinyl leukotrienes are lipid mediators known for their potent bronchoconstrictive properties, and have been associated with asthma and inflammation. Despite this association, there have been no report on the expression of cysteinyl leukotrienes or their receptors in human T cells and little knowledge on the exact role of leukotrienes. In the data we obtained from microarray studies using human T cells, we have identified the expression of CYSLTR1 in T cell subsets.

The aim of this study was to examine the expression of CYSLTR1 in T cell subsets observed in microarray studies and characterise the function of CYSLTR1 in human *in vitro*-differentiated Th2 cells. To achieve this, we performed a series of functional assays such as calcium, cyclic AMP and chemotaxis assays, but also microarrays to identify the effect of CysLTs on the expression of signature genes in Th2 cells.

1.5 Hypothesis

Based upon preliminary observations that CysLTR1 mRNA may be selectively expressed by human Th2 cells, we hypothesise that:

Human Th2 cells are a target of cysteinyl leukotrienes via selective expression of functional CysLTR1.

To test this hypothesis, we have performed several experimental tests:

1. Measurement of CysLTR1 mRNA levels in human Th1 and Th2 cells
2. Measurement of calcium flux in Th1 and Th2 cells in response to cysteinyl leukotrienes
3. Measurement of cyclic AMP signalling in Th1 and Th2 cells in response to cysteinyl leukotrienes
4. Measurement of chemotaxis in response to cysteinyl leukotrienes

CHAPTER 2
MATERIALS & METHODS

Chapter 2 Materials & methods

2.1 Materials

General laboratory chemicals were purchased from Invitrogen unless otherwise noted. Antibodies were purchased as described in tables 2.1 and 2.2.

2.1.1. Reagents

All cysteinyl leukotrienes, their antagonists (MK-571, Montelukast, Pranlukast) and PGD2 were purchased from Cayman Chemicals. SDF1α and L-cysteine were purchased from Sigma.

2.1.2. Kits

miRNeasy mini Kit was purchased from Qiagen. RNA 6000 Nano and Pico Kits was purchased from Agilent Technologies. HitHunter Assay cAMP XS+ and FLIPR calcium assay kit were purchased from Molecular Devices. Ambion WT Expression Kit was purchased from Invitrogen.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 HEK 293 cells

HEK (Human Embryonic Kidney) 293T adherent cells were obtained from Susan John (King's College London) and were maintained at 1 to 2 x 10^5 cells/ml and cultured in pre-heated Dulbecco's Modified Eagle Medium (DMEM) containing 2mM L-Glutamine supplemented with 10% Fetal Bovine Serum (FBS, Sigma) and 10% PSG (penicillin, streptomycin, glutamine) at 37°C, 5% CO_2 in humidified air. Cells were routinely

passaged at 1:8 to 1:10 dilution when reaching confluence. The approximate cell number for 100% confluence for this cell line in a T75 flask is 1×10^7 cells.

2.2.1.2 Primary human T cells

The studies described in this thesis were approved by the Research Ethics Committee of Guy's Hospital with informed consent. The donors used in this study were non-atopic, non-asthmatic male individuals (25-40 years) with no other chronic or acute illness at the time of venepuncture.

Primary human naïve T cells were cultured in RPMI/10%FCS/PSG medium and expanded in fresh medium approximately every 2 days. Cells were re-stimulated every 7 days with anti-CD3/anti-CD28 under the same conditions.

For Th1 differentiation, rIL-2 (50 units/ml; Novartis, Horsham, U.K.), rIL-12 (2.5 ng/ml; R&D Systems, Abingdon, Oxfordshire, U.K.), anti-IL-4 (5 μg/ml; clone MP4-25D2; BD Biosciences), and anti-IL-10 (5 μg/ml; clone JES3-9D7; Invitrogen) were added.

For Th2 differentiation, rIL-2 (50 units/ml; Novartis, Horsham, U.K.), rIL-4 (12.5 ng/ml; R&D Systems), anti-IFNγ (5 μg/ml; clone B-B1; Invitrogen) and anti-IL-10 (5 μg/ml; clone JES3-9D7; Invitrogen) were added. Cells were incubated at 37°C, 5% CO_2. A schematic diagram of these conditions is shown below on Figure 2.1. After 4 days, cells were removed from the coated plate and expanded under the same conditions as above in the absence of anti-CD3 and anti-CD28. Cells were then re-stimulated every 7 days with anti-CD3/anti-CD28 under the same conditions.

For time-courses with LTD_4, cells were cultured as described with 100 nM LTD_4 added to the cells. For Day 21 time-courses prior to microarrays, all cells were incubated with and without 100 nM LTD_4 with 3 nM L-cysteine, an inhibitor of dipeptidase (the enzyme responsible for converting LTD_4 to LTE_4), for a period of 24 hours. Samples with and without LTD_4 were taken at 30 minutes, 4 hours, and 24 hours. Samples were spun down using a micro centrifuge for 10 min at 13000 g, the supernatant removed and the pellets frozen at -80°C.

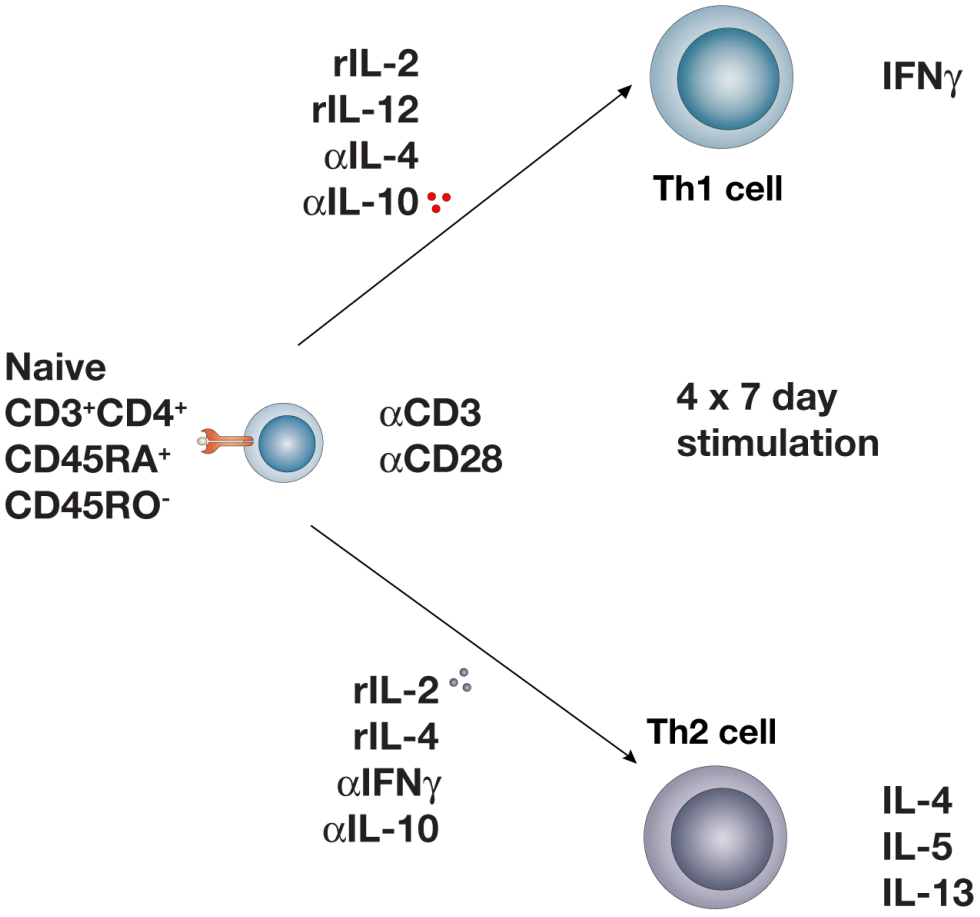


Figure 2.1. Human Th1/Th2 *in vitro* culture system (Cousins et al. 2002a).

Antibody	Company	Cat No
CD3	ECACC	Grown in house
CD28	Sanquin	M1650
CD45 RO	BD Pharmingen	555491
IFN-γ	ECACC	Grown in house
IL-4	ECACC	Grown in house
IL-10	ATCC	Grown in house

Table 2.1 Antibodies used for the culture and differentiation of primary human T cell *in vitro*.

2.2.2 Human T cell isolation

Human naïve T cells were isolated from 120 ml of human blood from healthy volunteers, which was anti-coagulated with heparin (1000U/ml), and mixed with an equal volume of PBS before being layered onto Lymphoprep (Axis-shield, Kimbolton, U.K.) to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were counted and resuspended in 5 ml of PBS/2%FCS. To isolate CD4 positive (CD4+) T-cells a CD4 Positive isolation Kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, CD4 Dynabeads were added to PBMCs at a ratio of 4 beads per cell and incubated in the cold room (4°C) for 20 min on a shaker. $CD4^+$ cells were then selected using the magnetic particle concentrator (MPC) and washed three times with ice cold PBS/2%FCS. The Dynabeads were removed using DETACHaBEADS with a 45 min incubation at room temperature followed by magnetic selection on the MPC. Naïve $CD45RA^+$ T cells were purified from $CD4^+$ T cells by depletion of $CD45RO^+$ cells, using mouse anti-human CD45RO at 10μl/$1x10^7$ cells amount/cell (UCHL1; BD Biosciences, San Jose, CA). Cells were then washed twice in PBS/2%FCS and incubated for 20 min in the cold room with pre-washed rat anti-mouse pan IgG Dynabeads (Invitrogen) according to manufacturer's instructions. After magnetic depletion of $CD45RO^+$ cells, unbound cells ($CD45RA^+$) were removed, centrifuged at 200 g for 10 min at 4°C and resuspended at $1x10^6$/ml in sterile RPMI/10%FCS/PSG, before being plated on tissue culture plates pre-coated with anti-CD3 and anti-CD28. Plates were coated with anti-CD3 (1 μg/ml; clone OKT3) and anti-CD28 (2 μg/ml; clone 15E8; Sanquin Reagents, Amsterdam, The Netherlands) in HBSS for 1.5 hrs at 37°C depending on cell number on either 24- or 48- well plates, prior to plating.

2.2.3 Flow cytometry

Human T cells were assessed for surface and intracellular cytokine expression prior to their use in calcium assays, cyclic AMP assays, chemotaxis assays and their re-stimulation. Approximately 2 x 10^6 cells were harvested, washed with fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline (PBS) containing 0.25% Bovine Serum Albumin (BSA) and 10% sodium azide) and centrifuged at 200 *g* for 5 minutes before being incubated with different antibodies at different concentrations. Cells were subsequently washed in FACS buffer, spun down for 5 minutes and resuspended in 500 µl of FACS buffer prior to flow cytometry analysis. Flow cytometry was performed using a FACScalibur (BD Biosciences, Oxford, UK). For analysis, a total of 10,000 live cell events were collected and gated based on cell size and granularity using forward and side scatter parameters. FITC- or AlexaFluor 488- labelled antibody staining was read on the FLH-1 channel, PE-labelled antibody on the FLH-2 channel and AlexaFluor 647- or APC-labelled samples on the FLH-4 channel. Results were analysed using BD Biosciences CellQuest^{TM} Pro software, version 5.2 and FlowJo Flow Cytometry Analysis Software for Macintosh version 9.3.2. Dead cells were excluded by forward and side scatter. A list of antibodies can be seen in **Table 2.2**.

2.2.4 Surface and intracellular staining of Th1 and Th2 cells

Th1/Th2 cells from *in vitro* culture were stained on the surface and intracellularly on Day 7, 14, 21 and 28. Between 500 µl and 1 ml of each T cell population was stimulated for 4 hours with PMA (5 ng/ml; Sigma-Aldrich, St.Louis, WO) and ionomycin (500 ng/ml; Calbiochem, San Jose, CA) at 37°C. After 2 hours of the 4-hour incubation,

monensin (2 μM; Sigma) was added to the stimulated and unstimulated T cells and incubated at 37°C. Following the 4 hours incubation, cells were harvested and 7-amino-actinomycin D (7-AAD; 4 mg/ml; Sigma) was added to unstimulated and stimulated cells, which were then transferred to FACS tubes and incubated on ice for 10 min. Cells were washed with FACSFlow (BD Biosciences) and processed for intracellular cytokine staining with the BD Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions. 500 μl BD Cytofix/Cytoperm (Cat. No. 554722) were added to each tube, and each tube was vortexed and left to incubate for 20 min on ice. Cells were then washed twice in FacsFlow and the pellet was re-suspended in BD Perm/Wash Buffer (Cat. No. 554723), and left on ice while preparing the antibodies for staining for approximately 30-45 min. Antibodies were then added to the tubes, followed by the cells, and incubated on ice for 30 min in the dark. Cells were washed once in Perm/Wash and once in FACS Flow and re-suspended in 250 μl of FACS Flow for FACS analysis. All antibodies dilutions were prepared in Perm/Wash buffer. A list of antibodies can be seen in **Table 2.2**.

The remaining cells from the culture were counted using 50 μl of cells and 10 μl propidium iodide (10 μl/ml) and stained extracellularly with different antibodies. Cells were then centrifuged and re-suspended in fresh RPMI/10%FCS/1%PSG medium at a 1 million cells/ml concentration, before being plated on a freshly coated plate with HBSS, anti-CD3 (1 μg/ml; clone OKT3) and anti-CD28 (2 μg/ml; clone 15E8; Sanquin Reagents, Amsterdam, The Netherlands) and suitable cytokines and placed at 37ºC until Day 3, 12, 24. A list of antibodies can be seen in **Table 2.2**.

Antibody	Fluorochrome	Species	Company	Cat No
CD4	FITC	Mouse anti-human monoclonal	BD Pharmingen	555346
	APC	Mouse anti-human monoclonal	BD Pharmingen	555349
CD8	PE	Mouse anti-human monoclonal	BD Pharmingen	555367
CD45RA	FITC	Mouse anti-human monoclonal	BD Pharmingen	555488
CD45RO	PE	Mouse anti-human monoclonal	BD Pharmingen	555493
IFNγ	Alexa Fluor 488	Mouse anti-human monoclonal	In-house	
IL-4	PE	Mouse anti-human monoclonal	BD FastImmune	340451
	APC	Rat anti-human monoclonal	BD Pharmingen	554486
IL-5	APC	Rat anti-human monoclonal	BD Pharmingen	554396
IL-13	PE	Rat anti-human monoclonal	BD Pharmingen	554571
CysLTR1		Rabbit anti-human polyclonal	Novus Biologicals	NLS1317
		Rabbit anti-human polyclonal	Cayman Chemicals	120500

Table 2.2 Antibodies used for surface and intracellular staining using flow cytometry.

2.2.5 Measurement of calcium

Cultured human Th1 and Th2 cells were tested for their calcium signalling when fully differentiated at Day 21 and 28 in response to cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4. Cells were re-suspended at 200,000 cells/well in 100 μl RPMI/2%HEPES and plated on to a 96-well black-wall, clear flat-bottom assay plate (Costar), and an equal volume of loading buffer (Component A with 1X HBSS Buffer; FLIPR Calcium 4 Assay Kit; Molecular Devices) was added. The plate was incubated for 1 hour at 37°C and 5% CO_2. After incubation, the plate was centrifuged at 200g for 5 min and transferred directly to a FlexStation 3 Microplate Reader. Controls included a negative medium control of RPMI/2% HEPES, and a positive control SDF-1/CXCL12, and all wells were done in triplicates. Cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 were used at various doses ranging from 0.1, 1, 10 and 100 nM. Concentrations for positive controls PGD2 and SDF1α were used at various doses ranging from 0.1, 1 and 10nM, and 10 nM, respectively. Where indicated, the selective $CysLTR_1$ inhibitor MK571 was used at 100 nM, montelukast and zafirlukast were used at 10 nM concentrations, and added prior to adding the ligand. Pertussis toxin (PTX), a toxin produced by the bacterium *Bordetella pertussis* which we used to identify the $CysLTR_1$ G protein signalling pathway, was used in some of our experiments at a concentration of 0.1 mg/ml (PTX, Sigma) for a 16 to 24-hour incubation period to inhibit Gαi. Where indicated, EDTA and thapsigargin (THP) were used to determine the origin of calcium in our assays, at concentrations of 2.5 mM and 1μM, respectively, and added prior to adding the ligand. Results were analysed using SoftMax Pro Software (Molecular Devices), and data are

shown as a percentage of maximal response. All experiments were replicated at least three times and performed in triplicate.

2.2.6 Measurement of cyclic AMP

Intracellular cyclic AMP (cAMP) levels were measured by luminescence with the HitHunter cAMP XS+ assay (DiscoverRX, Birmingham, United Kingdom). The assay was carried out according to the manufacturer's protocol for a 3-reagent addition. Human Th2 cells were tested for their cAMP signalling when fully differentiated at Day 21 and 28 (on the same day as the calcium and migration assays). Th2 cells were suspended in PBS at a concentration of 10 x 10^6/ml and warmed to 37°C, and 1 nM 3-isobutyl-1-methylxanthine (IBMX, Sigma) was added to inhibit cAMP phosphodiesterases. The Th2 cell suspension (2 x 10^5 cells/well) was loaded into a 96-well plate, and 20 µM forskolin were added to stimulate cAMP induction along with varying concentrations of LTD_4 ranging from 0.1 to 100 nM, as indicated in the results section. Where indicated, 100 nM MK571 was added 5 minutes prior to adding the ligand. Plates were incubated at 37°C for 15 minutes before cells were processed according to the kit's instructions, and cAMP signal was detected by means of luminescence measured 4 hours after cell lysis. Luminescence was detected by using a Flexstation 3 (Molecular Devices), and analysis of cAMP concentrations was performed with SoftMax Pro software (Molecular Devices). Data are shown as a percentage of maximal response. All experiments were replicated at least three times and performed in triplicate.

2.2.7 Migration assay

Chemotaxis assays were carried out using 24 well 6.5mm Transwell® plates with a polycarbonate membrane insert filter with pore size of 5.0 μm (Corning, 3421).

In vitro cultured human Th2 cells were resuspended at $1x10^6$/ml in RPMI buffer and rested for 4 hours in a low rIL-2 concentration (25 units/ml; Novartis, Horsham, U.K.), then harvested, washed in PBS and resuspended at 500,000 cells/100 μl in chemotaxis buffer (RPMI + 20 nM Hepes + 0.5% ultrapure BSA (Sigma. A7638). 600 μl chemotaxis buffer with or without chemokine or leukotriene of interest were loaded on the bottom of the transwell. Each leukotriene (LTD_4, LTE_4, positive control SDF1) concentration (at 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 nM) or buffer alone control was tested in duplicate. Using tweezers, the top of transwell was placed in the bottom well and cells were left to chemotax for 2 hours in a 37ºC and 5% CO_2 incubator. After 2 hours, the plate was placed in the refrigerator for 15 minutes. The top of the transwell was then removed and 20,000/20 μl beads (polystyrene 15 μm beads, Polysciences, 18328) were added per well and the mixture of cells and beads was transferred to a FACS tube. The number of cells that migrated through the filter was determined by flow cytometry by setting SSC to log scale to visualise the beads on scale, then setting a gate around beads and acquiring 10,000 beads per tube. Cell numbers were processed and plotted using Prism software. A Chemotactic Index (CI) was calculated as the ratio of migration in any given sample to the basal migration to buffer alone. A CI value of 1 therefore represents no chemotaxis above basal migration to buffer alone.

2.2.8 Proliferation assay

200,000/200µl Th1 and Th2 cells were seeded per well on a 24-well plate in RPMI complete medium. Some of the cells were plated with 100 nM LTD_4 and some without. After 24 hours, 10 µl/well (1:2) 3H-Thymidine were added to the cells. Cells were then incubated for 18 hours at 37°C. The more cell divisions (or the higher the proliferation rate) the more radioactivity will be incorporated into DNA. Following incubation, filter paper was used over the plate and the cells were harvested by washing the cells with distilled water several times. The cell fragments and DNA were passed through a filter membrane (glass fibre). The filter membrane was dried and transferred to a white plate containing 25 µl microsin (scintillation fluid), before being analysed on a scintillation counter to measure the amount of radioactivity.

2.2.9 RNA extraction

For RNA extraction of fully differentiated Th1 and Th2 resting and activated cells, cells were harvested at Day 21 or Day 28, washed in FACS flow and PBS and spun down at 1200 rpm to obtain a pellet. Cells for RNA isolation were snap-frozen in liquid nitrogen and stored at -80°C. Total cellular RNA was isolated from resting and activated T cells using the miRNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Briefly, cells were thawed out and lysed using 700 µl Trizol (Invitrogen) before being vortexed to homogenize. 140 µl of chloroform were added to each lysate and phases were mixed by vigorous shaking for 15 seconds before placing the tube at room temperature for 2-3 minutes. Phases were separated by centrifugation at 12,000 *g* for 15 min. The upper aqueous phase containing the RNA was pipetted out, placed in a new tube and mixed vigorously with 1.5 volumes of 100% ethanol, before being

transferred onto an RNeasy Mini column. Further steps were carried out according to the manufacturer's manual. RNA was eluted in 80 µl and mixed with 10 µl 10x buffer and 10 µl TurboDNase (TURBO DNase Kit, Ambion), and incubated for an hour at 37°C. 100 µl phenol:chloroform:isoamyl alcohol (25:24:1) (pH 5.2, Invitrogen) were added to the samples. The samples were vortexed for 20 seconds before centrifuging at 12,000 *g* for 15 min at room temperature to allow phase separation. The upper aqueous phase was carefully removed without disturbing the interface, or phenol phase, before adding 1.5 volumes 100% ethanol (approximately 150 µl) and transferring the samples to RNeasy Mini column. The samples were then washed according to the manufacturer's manual. The samples were eluted in 30 µl RNase-free water (1 min, 10,000 *g*, 20°C). RNA concentration was measured at 260 nm with a NanoDrop spectrophotometer (Thermo Scientific). RNA integrity was determined on a RNA 6000 Nano LabChip (RNA 6000 Nano Kit, Agilent Technologies) using the Agilent Bioanalyser 2100 machine (Agilent Technologies), according to the manufacturer's instructions.

2.2.9.1 Reverse transcription

Briefly, RNA samples were adjusted to 250 ng and 4 µl of random hexamer (0.1µg/µl pd$(N)_6$; Amersham) was added to each sample in a 22 µl final volume of water. Using the RevertAid First Strand cDNA Synthesis Kit, samples were incubated at 70°C for 5 min before being placed on ice. 16 µl of reaction mix (see below) were then added to each sample before an incubation of 5 min at 25°C.

Reaction Mix

8 µl 5x reaction buffer (Fermentas)

4 µl 10 nM dNTPs (Fermentas)

1 µl RiboLock (Fermentas)

2 µl dH_2O

2 µl Revert-Aid Reverse Transcriptase (Fermentas Life Sciences; 400 U/reaction) were added to each sample and the reaction incubated at room temperature for 10 min, before 42°C for 1 hour; followed by 70°C for 10 min, using a PTC-100 Thermal cycler. 260 µl dH_2O were then added to each sample. cDNA samples were stored at -80°C before use in real-time RT-PCR.

2.2.10 Microarrays

Following extraction of total cellular RNA and determination of RNA quality, as described earlier, RNA was reverse transcribed into cDNA, which was then *in vitro* transcribed into cRNA. For GeneChip U133 plus 2 arrays (Affymetrix), cRNA samples were prepared for microarray hybridization, according to the manufacturer's instructions using the Ambion MessageAmp Premier RNA amplification kit (Life Technologies). For GeneChip Human Gene 1.0 ST arrays (Affymetrix), cRNA samples were prepared for microarray hybridization according to the manufacturers instructions using the Ambion WT Expression Kit (Life Technologies). Fragmented cRNA was hybridized to GeneChip arrays at 45°C for 18 hours. Arrays were washed and stained with streptavidin-phycoerythrin, according to the manufacturer's instructions, on the GeneChip fluidics station 450 (Affymetrix). Fluorescent signals were detected

with the GeneChip scanner 3000. Images were analysed with the GeneChip Command Console Software (Affymetrix) to generate raw data as .cel files. Further analysis was performed with the Partek Genomics Suite (Partek, St Louis, Mo) by using the gene expression workflow to identify differentially expressed genes. Briefly, robust multichip average (RMA) pre-processing was performed, along with quantile normalization, and genes differentially expressed samples were identified by using the Partek ANOVA model. Statisical significance including false discovery rate analysis was performed using Partek and the Benjamini-Hochberg Step up procedure to correct for multiple comparisons.

2.2.11 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In order to confirm the results obtained by microarray analysis and to quantify gene expression, RT-PCR analysis was performed. Primer sets used for these experiments can be seen in Table 2.3. All primers and probe sets were tested for multiplexing with the endogenous control 18s rRNA primer set. Real-time PCR was carried out using 2X Master mix (Applied Biosystems), 18s rRNA (Applied Biosystems) 50 nM Primer, 83 nM Probe/reaction and 2.5 µl cDNA in a 15 µl reaction volume per well in 384 well plates. RT-PCR was carried out at 50°C for 2 min, 95°C for 10 min, 95ºC for 15 sec, and 60ºC for 1 min for 50 cycles using an ABI Prism 7900HT Thermal Cycler (Applied Biosystems). Results were analysed using SDS 2.4 Software (Applied Biosystems). Relative quantification was performed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001). Statistical analyses were performed by comparing gene expression levels relative to 18s rRNA levels by using a 2-way repeated-measures ANOVA with Bonferroni post tests in GraphPad Prism version 5.0a software (GraphPad, Inc, La Jolla, CA).

Real-time RT-PCR Reaction Mix

7.5 µl 2x Mastermix

0.5 µl FAM primer set

0.5 µl 18s VIC primer set

4.0 µl dH_2O

2.5 µl cDNA

2.2.12 Statistical Analysis

Statistical analyses were performed using GraphPad Prism or Partek GS. The statistical tests used are described in each section of the methodology or figure legend.

Gene	TaqMan® MGB Gene Expression Assay ID	Probe sequence
IL-5	Hs00174200_m1	CATAAAAATCACCAACTGTGCACTG
IL-4	Hs00929862_m1	CTTTGCTGCCTCCAAGAACACAACT
IFNγ	Hs00174143_m1	GAAATATTTTAATGCAGGTCATTCA
IL-9	Hs00174125_m1	GCGGGCTTGAATTCCTGTCCTGTGA
IL-13	Hs00174379_m1	CCCAGAACCAGAAGGCTCCGCTCTG
CYSLT1R	Hs00272624_s1	TTCAACGTACCATTCACCTTCATTT
CYSLT2R	Hs00252658_s1	CAGGTTAGTTGACCTTGCTGCAGTT

Table 2.3 Primers used in RT-PCR experiments.

2.2.13 Transfections

HEK293 cells were seeded at 3 x 10^6 cells/10cm plate with warm Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-Glutamine, 10% FBS and 10% PSG, on Day 0 and incubated for 24 hours at 37°C. On Day 1, 10 to 20 µg of construct DNA was used and the volume adjusted to 250 µl with sterile H_2O. 250 µl of 0.5 M Calcium Chloride solution ($CaCl_2$, Fluka) were mixed with the DNA and this was added dropwise on to 500 µl of 2x concentrated HEPES buffered saline (HBS, Fluka) while bubbling with a thin-tipped pastette. Tubes were left to stand for 30 min at room temperature, before being added dropwise to the plated HEK293 cells. Cells were incubated for 24 hours at 37°C. On Day 2, media was removed and replaced by 10ml of DMEM, and incubated for 24 hours. On Day 3, cells were harvested and stained with antibodies for FACS analysis or frozen for protein extraction and western blotting.

2.2.14 SDS-PAGE and Western blotting

2.2.14.1 Cell lysis

HEK293 transfectants were grown in 10 cm plates at 3 x 10^6 cells/10cm. DMEM medium was removed and cells were rinsed twice in ice-cold PBS. Cells were lysed in 150 µl lysis buffer (details below). The lysates were collected and boiled for 5 min at 100°C. To fragment genomic DNA, the lysates were sonicated on ice and centrifuged for 2 min at 10,000 *g*. The supernatants were aliquoted and stored at -20°C.

Lysis Buffer

Tris.HCl 50 mM pH7.5

EDTA 1mM

1% TritonX-100

In 10 ml 1 tablet protease inhibitors (Roche 11 836 170 001)

In 10 ml 1 tablet phosphatase inhibitors (Roche 04906845001)

2.2.14.2 Protein denaturation

Proteins were denatured according to manufacturer's instructions. Briefly, 20 μg protein were made up to a final volume of 19.5μl using lysis buffer. 3μl of reducing agent (NuPage® Sample Reducing Agent (10x), Invitrogen) and 7.5μl of sample buffer (NuPage® Sample Buffer (4x), Invitrogen) were added to the protein mix and samples were incubated at 95ºC for 10 min. Samples were loaded on to a NuPage ® 10% Bis-Tris Gel 1.0mm (Invitrogen) next to 3μl of MagicMark protein standard (Invitrogen). The gel was run in a gel tank filled with running buffer (50ml 20x running buffer in 1L water with 500μl NuPage ® antioxidants, all Invitrogen) at 180V for 1h30.

2.2.14.3 Western blotting

Protein was then transferred onto a membrane (Nitrocellulose Membrane Filter Paper Sandwich, 0.2μM pore size, Invitrogen) using an XCell II Blot Module (Invitrogen) containing two blotting pads, two filter papers, the gel, the transfer membrane, two filter papers and two blotting pads in that order. The transfer module was placed in

the chamber and the chamber filled with transfer buffer (50ml transfer buffer and 100ml methanol in 1L water with 1ml antioxidants, all Invitrogen), run at 30V for 1h.

The membrane was blocked in 5% milk in TBS-Tween (1 sachet Tris Buffered Saline with Tween ® 20, pH8.0, Sigma, in 1L of water) for 1h30 placed at room temperature on a plate rocker.

Membranes were then incubated with primary antibody anti-CysLT1R (polyclonal, Cayman Chemical Company) at 1:100, 1:1000 and 1:5000 dilutions in 5% milk in TBS-Tween, at 4°C overnight on a plate rocker. Membranes were washed three times for 10 min with TBS-Tween then incubated with a secondary goat anti-rabbit antibody (Southern Biotech) at a 1:5000 dilution in 5% milk in TBS-Tween, on a plate rocker at room temperature for 1h30. Membranes were then washed again three times for 10 min with TBS-Tween, developed for 5 min using the ECL Western Blotting Detection Kit (GE Healthcare), and developed using a COMPACT X2 developer (X-OGRAPH LTD) following 30 sec, 1 min, 5 min, 30 min exposures.

Membranes were then washed three times for 10 min in TBS-Tween and stripped using Restore ™ PLUS Western Blot Stripping Buffer (Thermo Scientific) for 15 min. They were washed again three times for 10 min in TBS-Tween and blocked for 1h30 with 5% milk in TBS-Tween, followed by an overnight incubation with primary antibody anti-GAPDH (GTX28245, GeneTex) at 1:25000 dilution in 5% milk in TBS-Tween. Membranes were washed again three times for 10 min then incubated 1h30 min with a secondary goat anti-mouse (IgG-HRP, Santa Cruz Biotechnology), followed by an additional three washes and then developed. Membranes were developed using the GE Healthcare ECL plus detection kit.

CHAPTER 3
RESULTS: EXPRESSION & SIGNALLING OF CYSLTR1

Chapter 3 Results – Expression & signalling of CYSLTR1

3.1 Introduction

Although a large amount of studies have searched to define the role of leukotrienes and their receptors in asthma, there is still limited knowledge about the effect of leukotrienes on cells in the human lung or their exact mode of action. Numerous studies have attempted to characterise the $CysLT_1$ receptor and determine its expression profile, as well as try and define its role in the immune system and its effect on its surrounding cells. We have previously described the initial cloning studies of the CYSLTR1 gene and the experiments performed to date examining CysLTR1 expression. The following section provides an overview of the experiments investigating the function of CYSLTR1 in humans and mice.

There have been extensive studies on the signalling pathway used by CysLT1R. CysLT1R is a known G protein-coupled receptor, characterised by the seven-transmembrane topology and its pathway of activation is therefore well illustrated.

When CYSLTR1 was cloned by two groups in 1999 (Lynch et al. 1999; Sarau et al. 1999) using a cognate ligand strategy for fishing for orphan GPCRs, the biochemical and pharmacological findings confirmed the receptor belonged to the G protein-coupled receptors family characterised by seven transmembranes spanning the surface of the cell. Both cysteinyl leukotriene receptors belong to the class A rhodopsin family, particularly to the purine receptor cluster of phylogenetically related receptors, including P2Ys, F2Rs and PAFRs (Fredriksson et al. 2003; Kroeze et al. 2003).

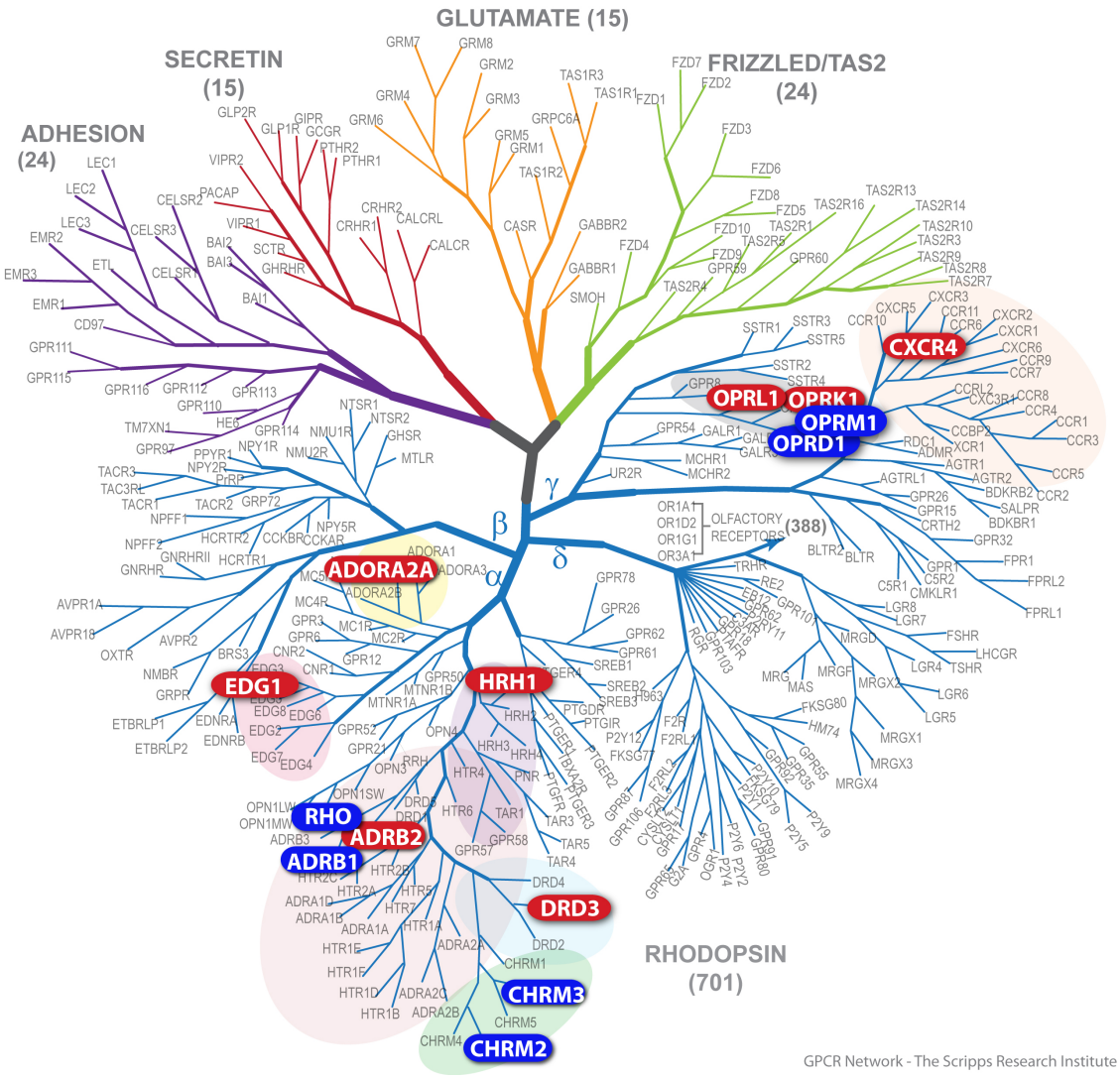


Figure 3.1 Phylogenetic trees of G protein-coupled receptors and discovered structures. Highlighted receptors indicate that the chemical structure of the GPCR has been solved. This map was taken from The Scripps Research Institute GPCR Network website http://gpcr.scripps.edu/(GPCR Network)

Several groups have therefore attempted to shed some light on the G protein-coupled receptor and its signalling G protein of choice, in various systems.

Monocytes and macrophages have been reported to respond to LTD_4 by calcium mobilisation. As mentioned previously, Thivierge *et al.* investigated the effect of IL-4 and IL-13 on calcium mobilization and the functional responsiveness of the cells to LTD_4. Treatment of monocytes with IL-13 or IL-4 lead to an increased calcium flux in response to LTD_4, but not LTB_4. A similar treatment of monocyte-derived macrophages led to an even larger increase than in monocytes. These responses were fully inhibited by antagonist MK571. Thivierge *et al* also investigated the effect of IL-13 and IL-4 on the migration of monocytes towards LTD_4, which we will get back to in the next chapter (Thivierge et al. 2001).

Cysteinyl leukotrienes also cause calcium flux in mast cells, which have the ability to induce calcium desensitisation in neighbouring resting mast cells via a CysLT paracrine signalling pathway (Di Capite and Parekh 2009). Strong calcium mobilization caused by LTD_4 was also found in bone marrow and peripheral blood CD34+ progenitor cells, with weaker effects observed using LTC_4 and LTD_4. This effect was blocked using $CysLT_1$ receptor antagonist MK571 (Bautz et al. 2001). Similar observations have been made in human basophils, and the effect blocked by antagonist zafirlukast (Gauvreau et al. 2005). In T cells, LTD_4 induces a robust calcium signal in *LatY136F* CD4+ T cells from mice but this was not reported in wild-type CD4+ T cells (Prinz et al. 2005).

It is known that both $CysLT_1$ and $CysLT_2$ receptors can couple to distinct types of G proteins. In recombinant systems, $CysLT_1$ receptor couples preferably to the Gαq/11 pathway, modulating inositol phospholipids hydrolysis and calcium mobilization,

whereas in native systems it has been found that the receptor generally activates a Gαi/o protein, sensitive to pertussis toxin, or both a Gαi/o and Gαq/11 pathway (Capra et al. 2005).

According to Lynch *et al*. (Lynch et al. 1999), treatment of HEK293 cells transfected with CysLT1R with pertussis toxin did not affect the Ca^{2+} responses produced by LTC_4, LTD_4, or LTE_4. Conversely in human cells, it was firstly reported using the human pro-monocytic differentiated U937 cell line, in which the receptor modulates calcium responses via Gαi/o and Gαq/11 (Capra et al. 2003; Saussy et al. 1989) but with the activation of the Ras-MAPK cascade entirely dependent on Gαi/o (Capra et al. 2004). Similarly in human acute monocytic leukaemia THP-1 cells, Hoshino et al (Hoshino et al, 1998) reported both a pertussis toxin-insensitive MAPK activation and a pertussis toxin-sensitive chemotactic response. More data confirmed these findings in airway smooth muscle cells (Ravasi et al. 2006) and mast cells (Mellor et al. 2001).

In human airway smooth muscle cells, Ravasi *et al.* found that LTD_4 was capable of inducing thymidine incorporation and potentiate EGF (epidermal growth factor)-induced proliferation. They showed that this proliferative effect of LTD_4 requires EGF-R phosphorylation via CysLT1R activation, PI3K and ROS production. They confirmed that the previously shown LTD_4-induced DNA synthesis is dependent on Gαi protein. Additionally, LTD_4 was also found to induce signalling pathways downstream of EGF-R, including the activation of Src-Ras-ERK1/2 signalling pathway (Ravasi et al. 2006).

In mast cells, Mellor *et al.* determined the functional expression of $CysLT_1$ receptor by stimulating the human mast cells with exogenous LTC_4 and LTD_4 with or without priming by IL-4. They found that both LTC_4 and LTD_4 elicited rapid, sustained elevations

in the levels of intracellular calcium with LTD_4 at lower concentrations than LTC_4. Priming with IL-4 had no significant effect on LTD_4-induced calcium but a 2-log increase in LTC_4-induced calcium flux, resulting in equal agonist effect of LTC_4 and LTD_4 following IL-4 priming. The addition of serine borate, which inhibits the extracellular conversion of LTC_4 to LTD_4, had no effect on the LTC_4-induced response; while the addition of chelating agent EGTA completely inhibited the sustained phase of the calcium response, suggesting that this phase was dependent on extracellular calcium. The group observed a response when using LTE_4 but the data was not shown. All calcium responses were inhibited by MK571, with or without priming with IL-4. Using BAY-u9773, a dual $CysLT_1R$ and $CysLT_2R$ antagonist, they found a weak calcium signal which was resistant to MK571 and which blocked subsequent reactivity to LTC_4 but not to LTD_4. The group also found that there was complete cross-desensitization of the hMCs by UDP to LTC_4, but not to LTD_4, which indicated that LTC_4 and UDP shared an MK571- sensitive receptor on human mast cells, the function of which was up-regulated by IL-4 (Mellor et al. 2002).

Despite extensive study, at the beginning of this project there was scant evidence to suggest that T cells were a target for CysLTs. The results below describe our finding that functional CYSLTR1 is expressed on human Th2.

3.2 Results

3.2.1 Microarray analysis reveals CYSLTR1 expression in human Th2 cells

We have previously reported a method to generate highly polarized human Th1 and Th2 cells from naïve precursors following *in vitro* differentiation and have used this model extensively to investigate the lineage specific functions of these cells (Cousins et al. 2002b; Sandig et al. 2007, 2009). To investigate novel lineage specific genes we performed a transcriptomic study comparing highly polarized human Th1 and Th2 cells.

Naïve CD4+ T cells were isolated from 120 ml of fresh whole human blood from healthy volunteers. PBMCs were firstly isolated using Lymphoprep, followed by a CD4+ isolation and memory CD45RO+ T cell depletion using anti-human CD45RO to obtain the naïve CD4RA+ T cells. Naïve CD4+ T cells were then cultured in plates pre-coated with anti-CD3 and anti-CD28 and a mix of cytokines. To induce Th1 cell differentiation, cells were cultured with rIL-2, rIL-12, anti-IL-4 and anti-IL-10. To induce Th2 cell differentiation, naïve cells were cultured with rIL-2, rIL-4, anti-IFN-γ and anti-IL-10. Cells were incubated at 37°C, 5% CO_2 and after 4 days were expanded under the same conditions without anti-CD3 and anti-CD28. Cells were then re-stimulated every 7 days and stained for extra- and intra-cellular cytokine expression. To identify novel specific molecules that may play a selective role in Th2 cell function, we have previously performed a microarray-based study, comparing Th1 and Th2 cells (Joanne McDonald, PhD thesis). Using 3 biological replicates of *in vitro*-differentiated human Th1 and Th2 cells, both in resting (R) and activated (A) states, a series of Affymetrix U133 plus 2 microarrays were performed to compare the gene expression profiles of Th1 R, Th1 A,

Th2 R and Th2 A cells and identify genes which were differentially expressed in those cell types. Differential gene expression analysis using ANOVA in Partek Genomics Suite identified 1627 genes (probe sets) that were differentially expressed between Th1 and Th2 cells (**Figure 3.2**). Examination of the gene list for differentially expressed cell surface molecules, which could potentially serve as lineage markers, identified CysLT1R as being highly and selectively expressed by Th2 cells. The Affymetrix U133 plus 2 microarray contains three probe sets that recognise CysLT1R. All three probe sets were reported as differentially expressed with higher expression in Th2 cells. As discussed earlier T-cells are not a recognised target cell type for CysLTs and therefore identification of CysLT1R expression by Th2 cells was somewhat surprising.

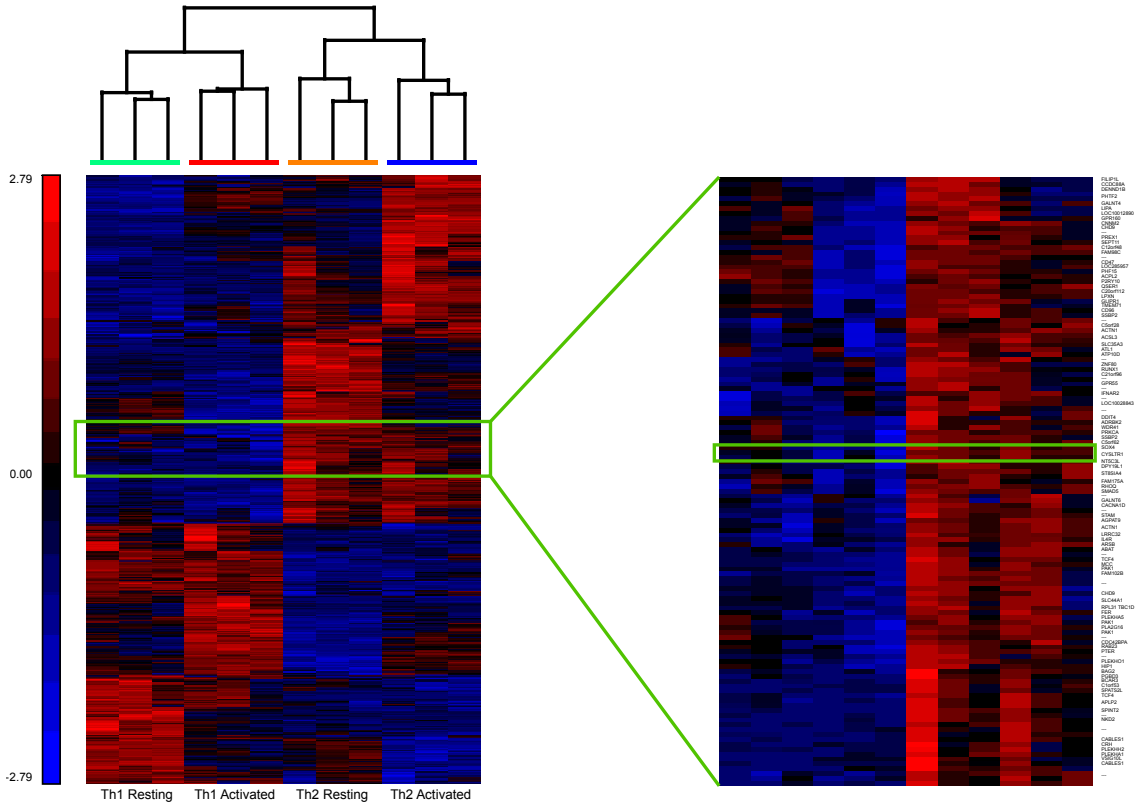


Figure 3.2 Hierarchical clustering of genes differentially expressed between human Th1 and Th2 cells. 1627 were differentially expressed between Th1 and Th2 cells with a 2-fold change and p-value <0.05 with FDR (ANOVA). CYSLTR1 is shown on the close up (right panel, p-value = 0.000587). 3 biological replicates of *in vitro*-differentiated Th1 and Th2 cells at Day 28 were used for gene expression microarrays to identify differential gene expression.

Gene Name	Probe set ID	Th1 Resting	Th1 Activated	Th2 Resting	Th2 Activated
CYSLT1R	230866_at	115.5	48.6	430.8	336.2
CYSLT2R	220813_at	14.4	14.8	18.1	17.2
GPR17	215225_s_at	17.2	16.6	14.5	14.4
P2Y12	224102_at	12.9	12.7	13.5	13.3

Table 3.1 Expression of selected genes in preliminary microarray analysis. CYSLT1R is highly expressed in Th2 resting cells in comparison to Th1 cells. Expression of other potential CysLT receptors such as CYSLT2R, GPCR17 and P2Y12 was undetectable by microarray analysis.

Expression of other potential cysteinyl leukotriene receptors CYSLTR2, GPR17 and P2YR12 was undetectable by microarray in both human Th1 and Th2 cell populations (**Table 3.1**).

To confirm the expression of CYSLTR1 by human Th2 cells, *in vitro* differentiation of Th1 and Th2 cells was performed using the model described earlier. Naïve CD4+ T cells were isolated from fresh whole blood and differentiated *in vitro* for 28 days using the cytokines and antibodies described above to induce Th1 or Th2 polarization. **Figure 3.3** shows the flow cytometry analysis of these *in vitro*-differentiated human Th1 and Th2 cells at Day 28 of polarization, in both resting and activated states. Intracellular cytokine staining of resting and activated Th1 and Th2 cells demonstrated that highly polarized Th1/Th2 populations were generated (**Figure 3.3**). Upon activation, at least 99% of the Th1 cells expressed IFN-ɣ with minimal Th2 cytokine expression. In contrast, the Th2 cells expressed negligible IFN-ɣ and a very high proportion express Th2-specific cytokines (IL-4, 42%, IL-5, 46%, IL-13, 85%).

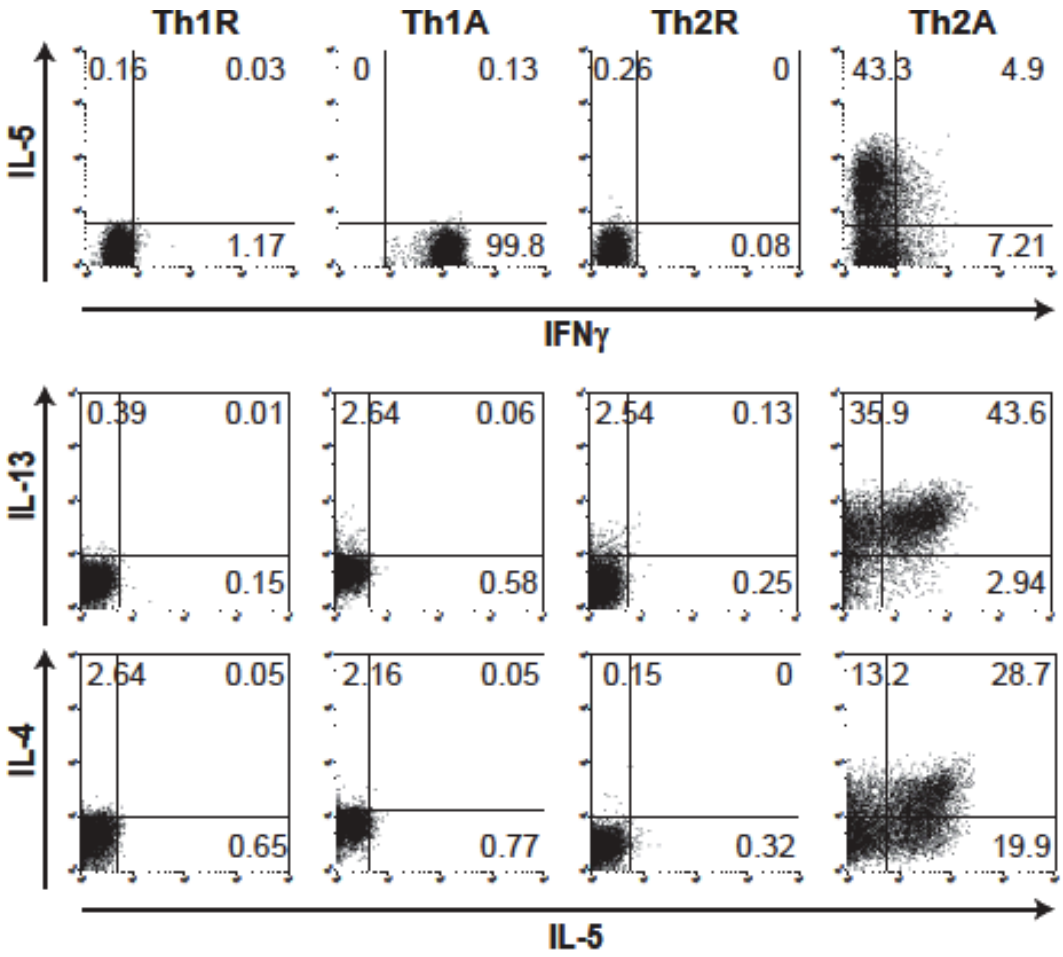


Figure 3.3 Intracellular cytokine staining of differentiated human Th1 and Th2 cells. Cells were either resting (R) or activated (A) for 4 hours with PMA (5ng/ml) and ionomycin (500ng/ml). Following the Th1/Th2 expression profile, Th1 express high levels of IFNɣ while Th2 cells express cytokines IL-4, IL-5 and IL-13, but no IFNɣ. Data shown are representative of 5 experiments with different donors.

Quantitative real-time RT-PCR of highly differentiated Th1 and Th2 samples confirmed that the Th1 and Th2 cells expressed high levels of transcripts for the hallmark cytokines IFN-ɣ (Th1) and IL-4, IL-5 and IL-13 (Th2) upon acute activation (**Figure 3.4**). Examination of CYSLTR1 mRNA expression by real-time RT-PCR confirmed that it is selectively expressed by resting Th2 cells with 6.5 fold higher levels than resting Th1 cells (**Figure 3.5**). The selective expression of CYSLTR1 was predominantly observed in cells prior to activation, with a substantial decrease in mRNA expression after acute activation of Th2 cells, suggesting a downregulation of the $CysLT_1$ receptor upon activation (**Figure 3.5**). Real-time PCR analysis also confirmed that CYSLTR2 mRNA was not expressed at high levels in either Th1 or Th2 cells (**Figure 3.5**). Interestingly, examination of CYSLTR1 mRNA expression during the time-course of differentiation revealed that expression increased substantially during Th2 differentiation (**Figure 3.6**). This data suggests that CYSLTR1 expression is acquired during Th2 differentiation rather than being expressed on naïve T cells and selectively lost during Th1 differentiation. This also indicated that expression of CYSLTR1 by Th2 cells is not merely caused by exposure to IL-4 since it requires at least three weeks of *in vitro* culture before observing high levels of expression (**Figure 3.6**). The genes adjacent to CYSLTR1, PGK1, TAF9B and ZCCHC5 were not differentially expressed between Th1 and Th2 cells suggesting that the Th2 selective expression of CYSLTR1 is not merely due to the gene being located in close proximity to other Th2 specific genes (**Table 1**).

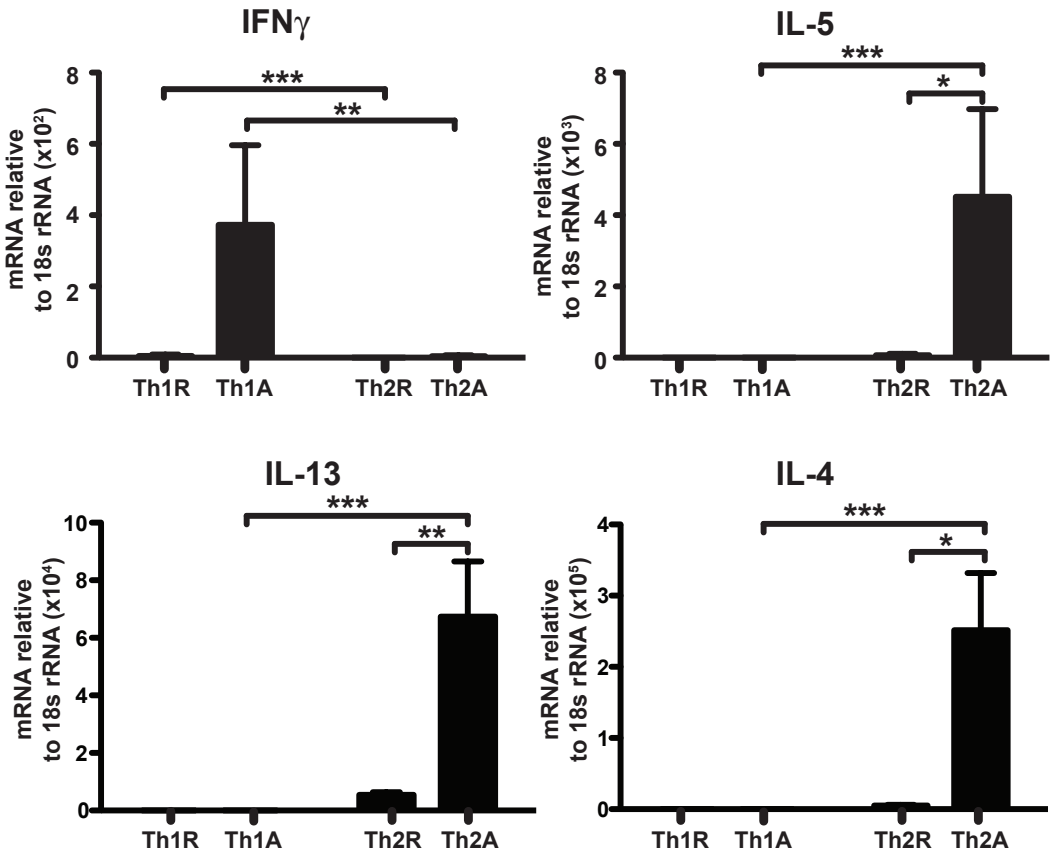


Figure 3.4 Real-time RT-PCR analysis of Th1 and Th2 cells for IFN-ɣ, IL-5, IL-13 and IL-4. IFNɣ is highly expressed by Th1A cells and IL-5, IL-4 and IL-14 by Th2A cells. These data show means ± SEMs of 5 experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, 2-way ANOVA with Bonferroni post test.

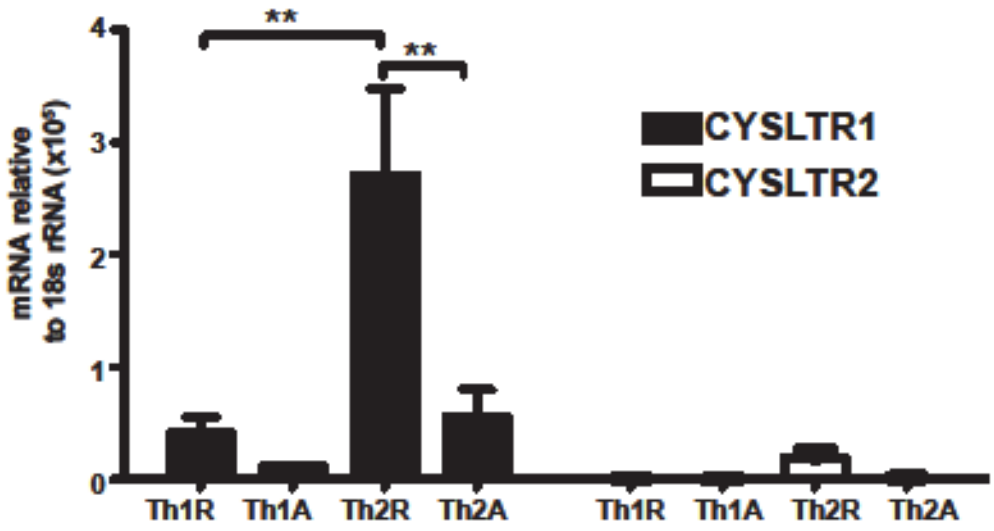


Figure 3.5 Real-time RT-PCR analysis of Th1 and Th2 cells for CYSLTR1 and CYSLTR2. CYSLTR1 is highly expressed by Th2R cells and downregulated upon Th2 cell activation. CYSLTR2 shows a low expression by Th1R/A and Th2R/A cells. These data show means ± SEMs of 5 experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001, 2-way ANOVA with Bonferroni post test.

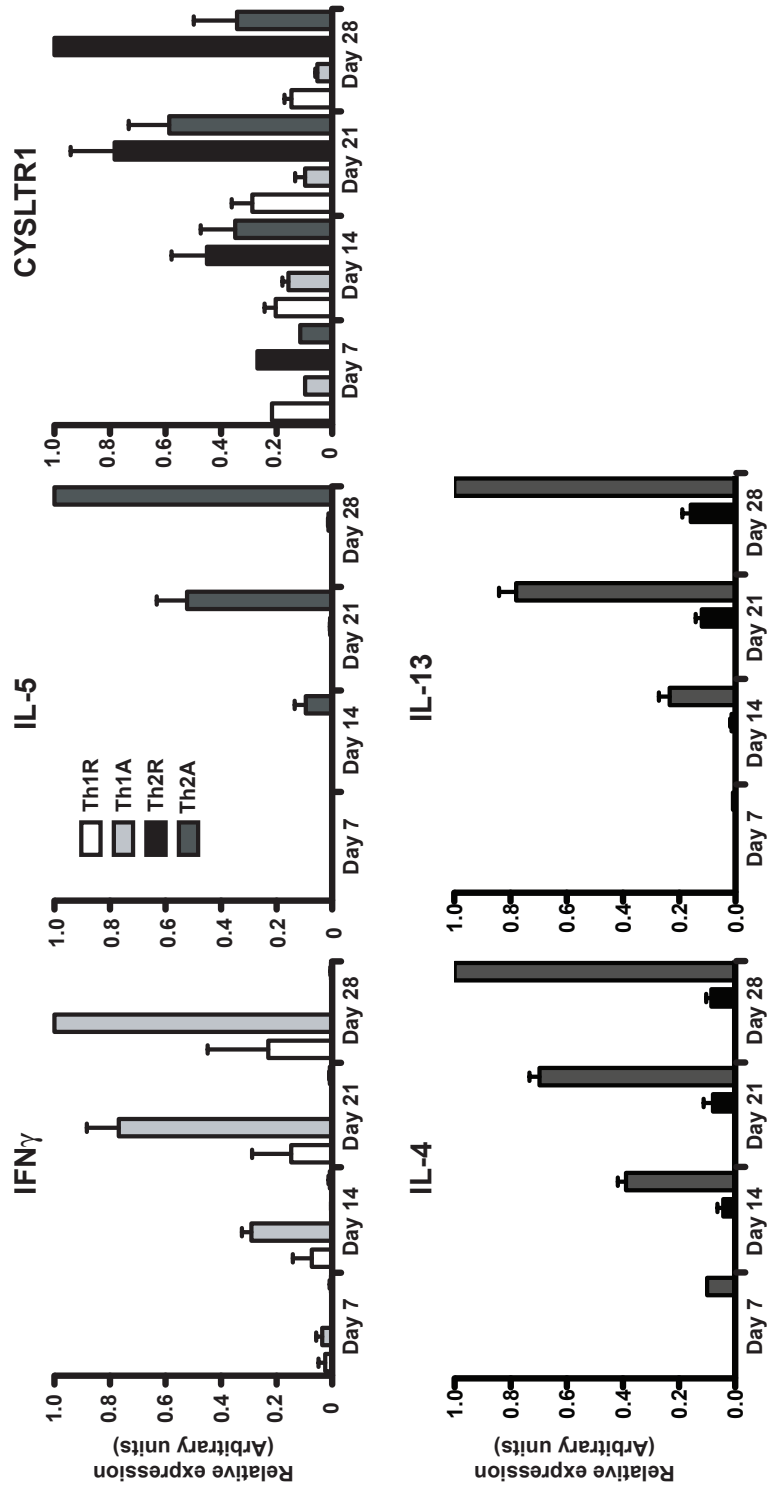


Figure 3.6 Time-course analysis of gene expression. Gene expression is shown during Th1/Th2 differentiation by using real-time RT-PCR. IFN-γ is highly expressed by Th1A cells and this expression increases with Th1 differentiation. IL-5 follows a similar profile and is highly expressed in Th2A cells. CYSLTR1 is highly expressed on Th2R cells and its expression increases with Th2 differentiation. IL-4 and IL-13 follow a similar pattern to IL-5 and are highly expressed in Th2A cells. These data show means ± SEMs of 5 experiments.

3.2.2 Detection of CYSLT1R protein in human cells

The results presented above confirm the initial microarray studies and show that mRNA for CYSLTR1 is selectively expressed in human Th2 cells when compared to Th1 cells. To demonstrate that the selective expression of CYSLTR1 mRNA results in CysLTR1 protein production in Th2 cells we attempted to examine CysLTR1 protein levels using both western blotting and flow cytometry.

Several groups have used these techniques to examine CysLTR1 expression in the past. Thivierge *et al.* showed expression of CysLTR1 protein by western blotting, using a rabbit polyclonal anti-human CysLTR1 antibody developed and characterised by Cayman Chemical (Thivierge et al. 2001). Expression was shown in monocytes and monocyte-derived macrophages after incubation with and without IL-13 and IL-4 (Thivierge et al. 2001).

Meliton *et al.* also used the CysLTR1 polyclonal antibody from Cayman Chemical and showed CysLTR1 protein expression in eosinophils and human polymorphonuclear leukocytes (PMNs) by western blotting. They found that eosinophils had significantly greater expression of 38 kDa CysLTR1 than PMNs. In their experiments, the western blotting membrane was probed with 2 µg/ml anti-CysLTR1 pAb and incubated with 1:3000 dilutions of goat anti-rabbit IgG conjugated with horseradish peroxidase. To detect the expression, the group used an enhanced chemiluminescence system (ECL, Amersham) (Meliton et al. 2010). Using two different antibody concentrations we were unable to detect CysLTR1 protein (**Figure 3.7**) in cell extracts isolated from HEK293 cells transfected with pCDNA3 overexpressing the human CYSLTR1 gene.

Examination of GAPDH expression on the same membranes indicated that the western blotting protocol was working satisfactorily.

Using the same rabbit polyclonal anti-human CysLTR1-antibody from Cayman Chemical we used for western blotting, we examined CysLTR1 protein expression in our samples. Cultured Th2 cells were tested for CysLTR1 expression and GAPDH was used as a positive control. CysLTR1 antibody (Cayman Chemical) was used at different concentrations such as 1:100 (data not shown), 1:1000 and 1:5000. Membranes were then incubated with a secondary goat anti-rabbit antibody (SouthernBiotech) at a 1:5000 dilution. Positive control anti-GAPDH (GTX28245, GeneTex) was used at 1:25000 dilution. Using the GE Healthcare ECL plus detection kit, we were unable to detect CysLTR1 expression in our samples at 10 min and 30 min exposure. However, positive control GAPDH was detected at 10 min exposure (**Figure 3.7**).

Using different anti-CysLTR1 antibodies, we also attempted to detect CysLTR1 by flow cytometry. Using Novus Biologicals rabbit polyclonal antibody (NLS1317) (**Figure 3.8(A)**), we were unable to detect surface CysLTR1 expression in Th1 or Th2 differentiated cells. Using Cayman Chemical rabbit polyclonal antibody (120500) (**Figure 3.8(B)**), we found similar results and failed to observe any intracellular CysLTR1 expression in Th1 or Th2 cells differentiated cells.

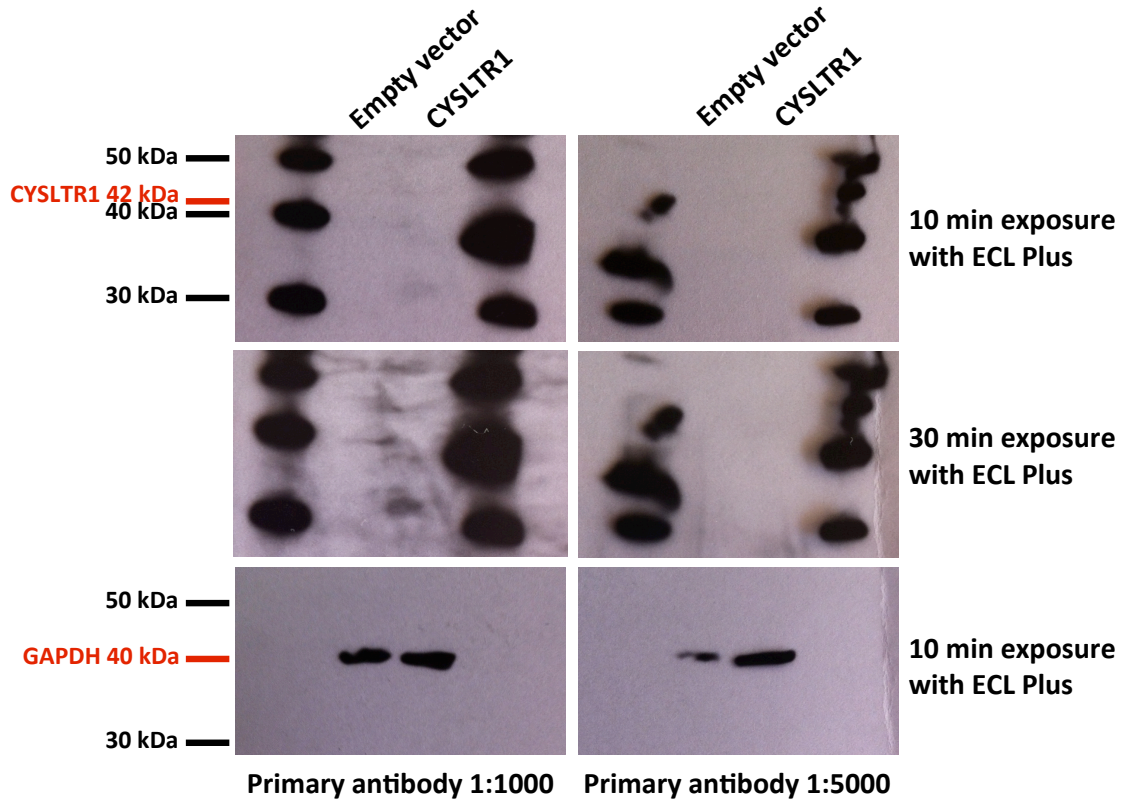


Figure 3.7 CysLTR1 detection by Western Blotting. No detection of CysLTR1 expression in CysLTR1-transfectants or empty vector using anti-CysLTR1 antibody (Cayman Chemical) at 1:1000 and 1:5000 and a goat anti-rabbit Ig-HRP secondary antibody at 1:5000. Positive control GAPDH could be detected in both samples. Data is representative of three experiments.

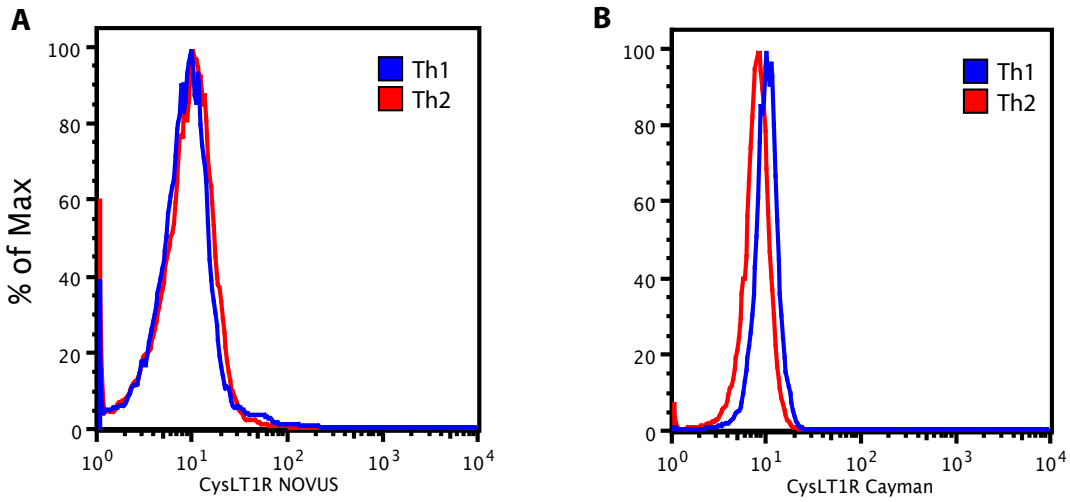


Figure 3.8 Flow cytometric analysis of human Th1 and Th2 cell expression of CysLTR1. Cells were differentiated in vitro for 28 days and were stained for CysLTR1 expression using protocols as described in the materials and methods. (**A**) Extracellular staining using Novus Biologicals rabbit polyclonal antibody (NLS1317). (**B**) Intracellular staining using Cayman Chemical rabbit polyclonal antibody (120500). Both were detected using chicken anti-rabbit Alexa Fluor 647.

Collectively our efforts to demonstrate CysLTR1 protein expression were unsuccessful no matter which approach we took. Given the recognised difficulty in detecting CysLT1R protein we decided to determine whether we could observe functional expression of CysLT1R in human Th2 cells.

3.2.3 Ca^{2+} mobilization through $CysLT_1$ receptor in resting Th2 cells

Following our finding of CYSLTR1 mRNA expression by human Th2 cells, we decided to assess whether CYSLTR1 mRNA gave rise to functional proteins via monitoring the intracellular Ca^{2+} mobilization through $CysLT_1$ receptor. Cysteinyl leukotrienes signalling through $CysLT_1$ receptor induce calcium flux in other cell types known to express the receptor. We determined whether the selective expression of CYSLTR1 mRNA observed in Th2 cells resulted in functional expression of CysLTR1 in these cells and whether *in vitro* differentiated human Th1 and Th2 cells were capable of Ca^{2+} mobilization in response to the known high affinity ligand for $CysLT_1$ receptor, LTD_4. The purpose of the experiment was to treat *in vitro*-differentiated Th1 and Th2 cells with LTD_4 and measure the intracellular calcium responses. For this we decided to use the FLIPR (Fluorescence Imaging Plate Reader) assay and optimise the assay conditions.

As per previous experiments, we used naïve CD4+ T cells isolated from fresh whole blood and cultured and differentiated them for 28 days using a combination of cytokines and antibodies to induce Th1 or Th2 polarization. Cultured human Th1 and Th2 cells were tested for calcium signalling when fully differentiated at Day 21 and 28 (when CysLT1R mRNA levels were maximal) in response to leukotrienes LTB_4, LTC_4, LTD_4 and LTE_4. Cells were resuspended in 100 μl RPMI/2%HEPES and plated on to a 96-

well black-wall, clear flat-bottom assay plate (Costar; Sigma), and an equal volume of loading buffer (Component A with 1X HBSS Buffer; FLIPR Calcium 4 Assay Kit; Molecular Devices) was added. The plate was incubated for 1 hour at 37°C and 5% CO_2. After incubation, the plate was centrifuged at 200g for 5 min and transferred directly to a FlexStation 3 Microplate Reader. Results were analysed using SoftMax Pro Software (Molecular Devices). Controls included a negative medium control of RPMI/2% HEPES, and a positive control SDF-1α, and all treatments were performed in triplicate. SDF-1α was used as a positive control as it is a chemokine known to attract and have an effect on both Th1 and Th2 cells (Siveke and Hamann 1998). Its receptor CXCR4 is also known to be preferentially expressed on naïve T cells (Bleul et al. 1997). Similar calcium flux results to the ones observed in our study were seen in a study by Drost *et al.*, using SDF-1α (Drost et al. 2012).

During optimisation experiments, we determined the optimum cell number by testing 50,000, 100,000 and 200,000 resting Th2 cells per well per assay. Simultaneously we tested increasing doses of LTD_4 such as 1 nM, 10 nM and 100 nM to determine whether calcium responsiveness was observed. As pictured in **Figure 3.9 A**, **B** and **C**, all cell numbers showed positive calcium responses to various doses of LTD_4, with optimum results seen when using 200,000 cells per well at 10 nM LTD_4 (**Figures 3.9**).

While optimising the FLIPR assay, we also tested the same cell numbers and doses of LTD_4 on *in vitro*-differentiated Th1 resting cells, in order to compare the calcium responses on the two types of cells (**Figure 3.10**). Based on previous experiments in which CYSLTR1 RNA levels were lower in Th1 cells than Th2 cells, we expected a much lower calcium flux response in Th1 cells. As seen in **Figure 3.10 A**, **B** and **C**, 200,000 Th1

cells and an LTD_4 dose of 10 nM also were the optimum conditions for the assay. However the calcium response in Th1 resting cells was considerably lower than in Th2 resting cells.

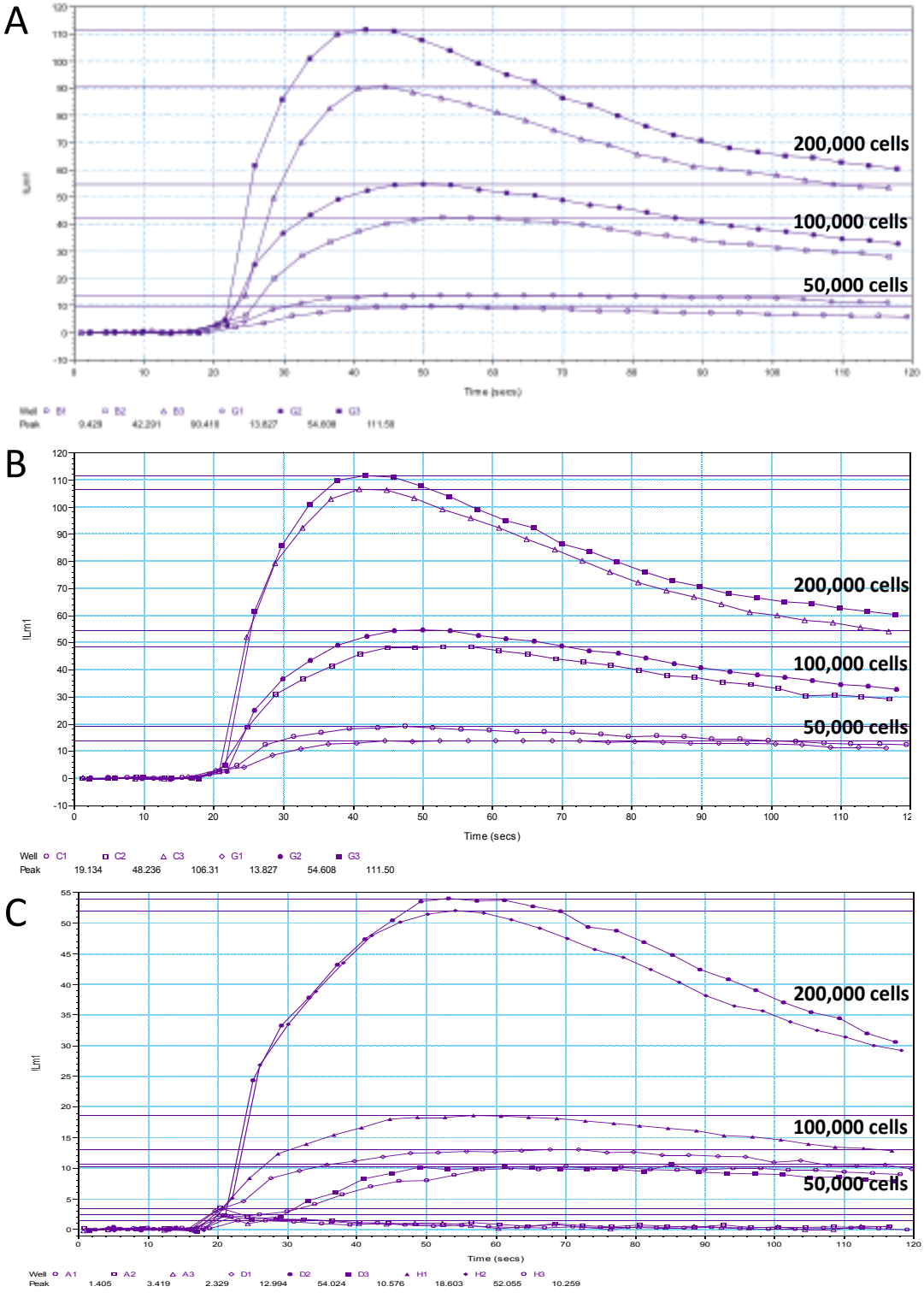


Figure 3.9 Optimisation of the FLIPR assay in Th2R cells. Raw data showing 50,000, 100,000 and 200,000 *in vitro*-differentiated Th2 resting cells and calcium flux in response to 1 (**A**), 10 (**B**) and 100nM (**C**) LTD_4. Using 1, 10 and 100nM LTD_4 resulted in a dose-dependent calcium response of approximately 100, 110 and 55 luminescence units, respectively. Data are representative of three experiments using at least 2 technical replicates.

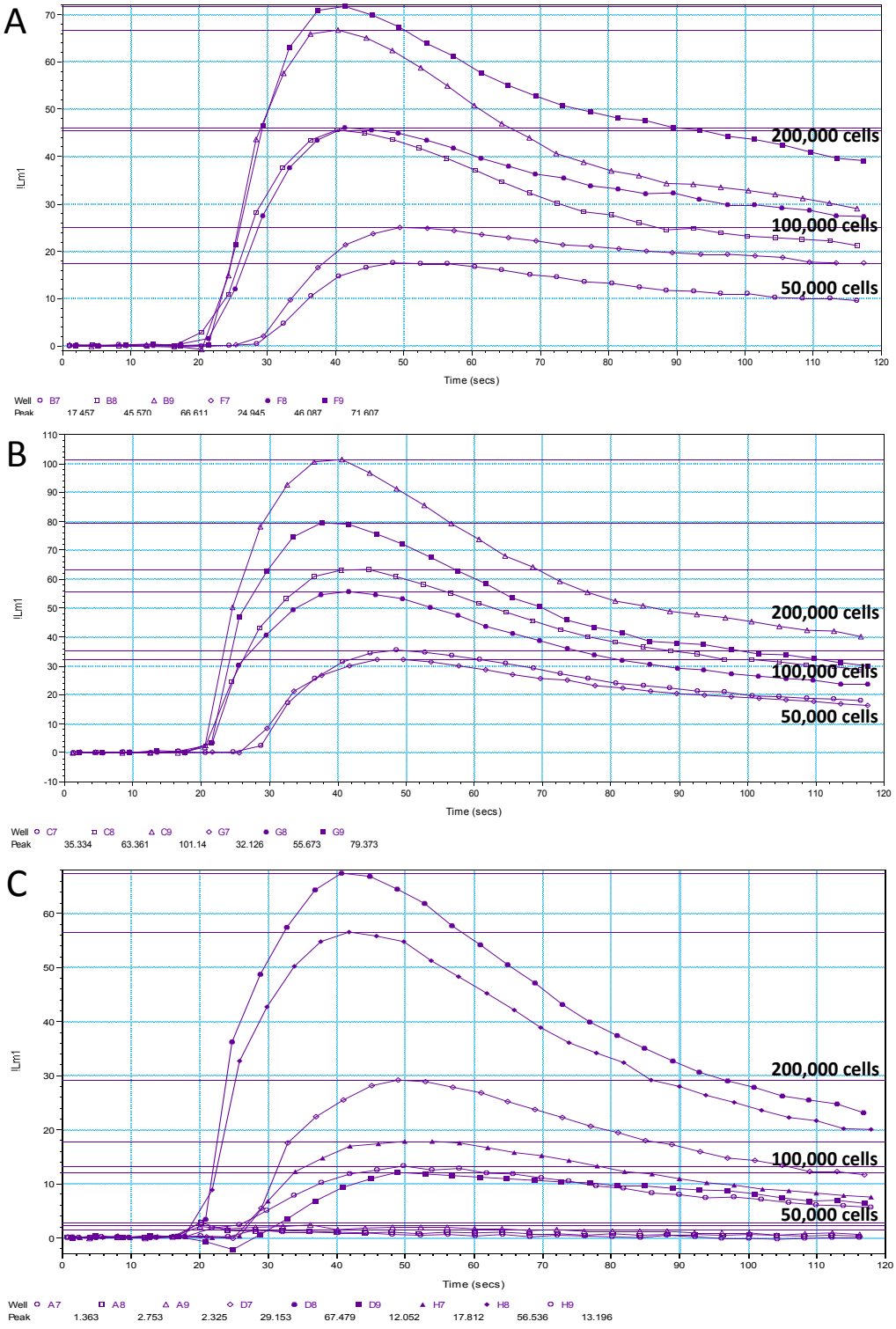


Figure 3.10 Optimisation of the FLIPR assay in Th1R cells. Raw data showing 50,000, 100,000 and 200,000 *in vitro*-differentiated Th1 resting cells and calcium flux in response to 1 (**A**), 10 (**B**) and 100nM (**C**) LTD_4. Using 1, 10 and 100nM LTD_4 on Th1 cells resulted in a dose-dependent calcium flux of approximately 65, 80 and 55 luminescence units, respectively. Data are representative of three experiments using at least 2 technical replicates.

Following the optimum results obtained using 200,000 Th2 resting cells per well with 10nM LTD_4, we tested the same conditions using the positive control SDF1α and the known CysLT1R antagonist MK571. We chose to use SDF1α as a positive control as it is a chemokine known to attract both Th1 and Th2 cells (Siveke and Hamann 1998), as well as spleen lymphocytes (Bleul et al. 1996), as mentioned earlier.

As observed in previous experiments, the calcium flux response to 10 nM LTD_4 in Th2 cells was very high with a calcium peak of approximately 110 fluorescence units, while SDF1α led to a moderate calcium response. The pre-incubation of Th2 cells with MK571 blocked the response to LTD_4 and the high calcium flux previously observed was fully inhibited. As a negative control, medium was added to Th2 cells and no calcium response was observed (**Figure 3.11)**.

Finally, we also ran the FLIPR assay using activated Th1 and Th2 cells. These cells had been activated using PMA and ionomycin for 4 hours prior to the assay. We tested the same conditions as for previous experiments on resting cells. As seen in **Figures 6A** and **6B**, Th1- (**A**) and Th2-(**B**) activated cells were tested at 50,000, 100,000 and 200,000 in cell numbers to check their response to 10 nM LTD_4, positive control SDF1α, and medium as negative control. Some wells were also pre-incubated with antagonist MK571 to inhibit the calcium response. Interestingly, the results observed using activated T cells peaked downwards and showed no calcium flux. A reason for this effect may be that as cells were activated, all receptors may have been internalised, leaving no receptor to respond to LTD_4 or SDF-1α and showing no calcium response.

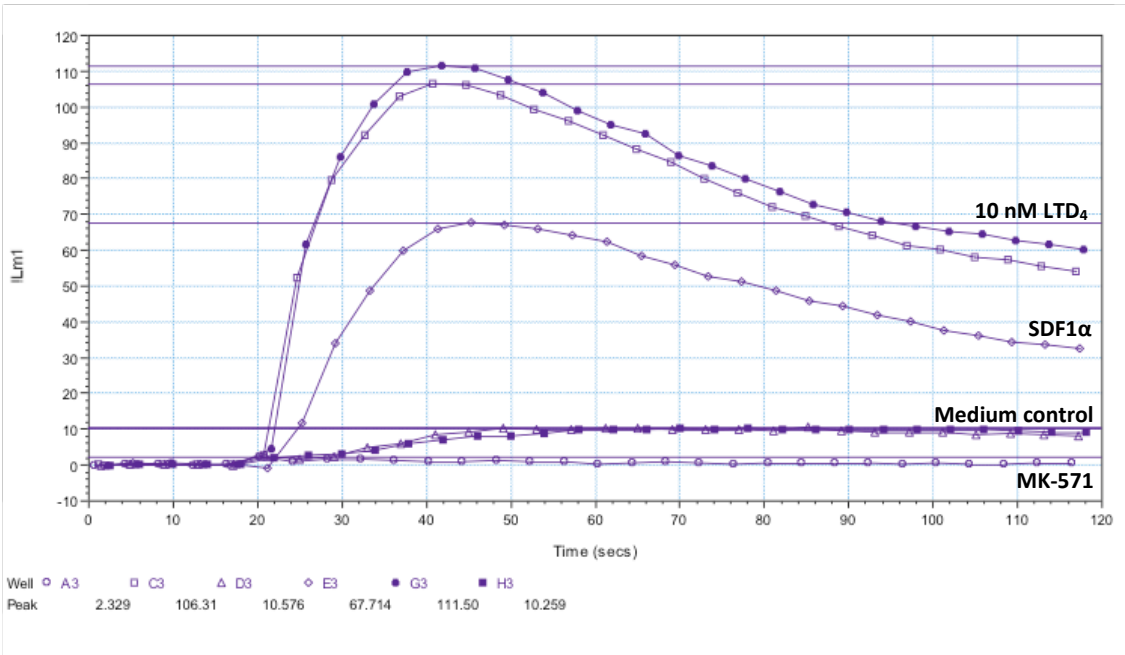


Figure 3.11 Optimisation of the FLIPR assay with positive control and inhibitor. Raw data showing 200,000 *in vitro*-differentiated Th2 cells and their calcium flux in response to 10 nM LTD_4, positive control SDF-1α and medium. Pre-incubation of Th2 cells with CysLT1R antagonist MK571 inhibited the calcium response completely. Data are representative of three experiments using at least 2 technical replicates.

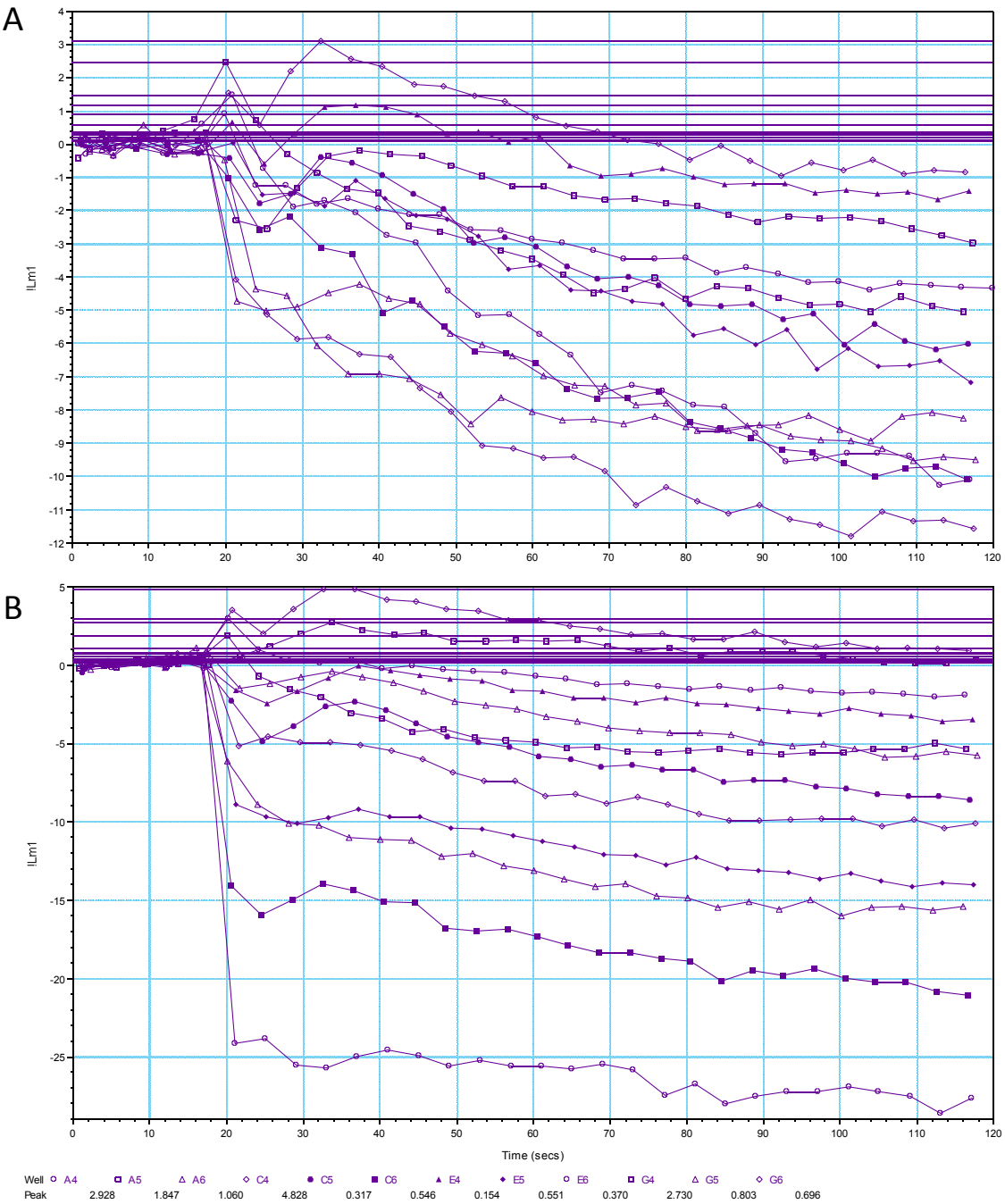


Figure 3.12 Calcium flux in activated *in vitro*-differentiated Th1A (**A**) and Th2A (**B**) cell. The graphs show a negative calcium flux in response to 10nM LTD_4, positive control SDF1α, and medium, using 50,000, 100,000 and 200,000 cells. Some samples were also pre-treatment with MK571 to inhibit the calcium response. Data are representative of three experiments using 2 technical replicates.

The results from the optimisation experiments lead us to conclude that 200,000 cells per well was optimal and all following experiments were performed with that amount of cells. We also decided that resting T cells were best for the assay. Once the FLIPR assay was optimised, we proceeded with experiments using 200,000 cells per well and using resting T cells. We first compared the calcium flux response in Th1- and Th2-resting cells using various doses of LTD_4. Consistent with the pattern of mRNA expression (see **Figure 3.6**), LTD_4 induced a very strong calcium mobilization in Th2 resting cells at concentrations similar to those reported for CysLTR1 in other systems with an EC_{50} of ~ 1nM (see **Figure 3.13**). A much weaker response was observed in Th1 resting cells (**Figure 3.13**), but not in activated Th1 or activated Th2 cells. The Ca^{2+} flux observed in both Th1 and Th2 cells was dose-dependent. This confirmed that human Th2 cells selectively express a functional receptor for LTD_4.

Treatment of Th2 cells with cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 showed that all three CysLTs induced calcium mobilization, with LTD_4 being the most potent, followed by LTC_4 and LTE_4 (**Figure 3.14**). This rank order of potency (LTD_4>LTC_4>LTE_4) strongly resembles previous studies on CysLTR1 in other cell types, suggesting that Th2 cells express functional CysLTR1.

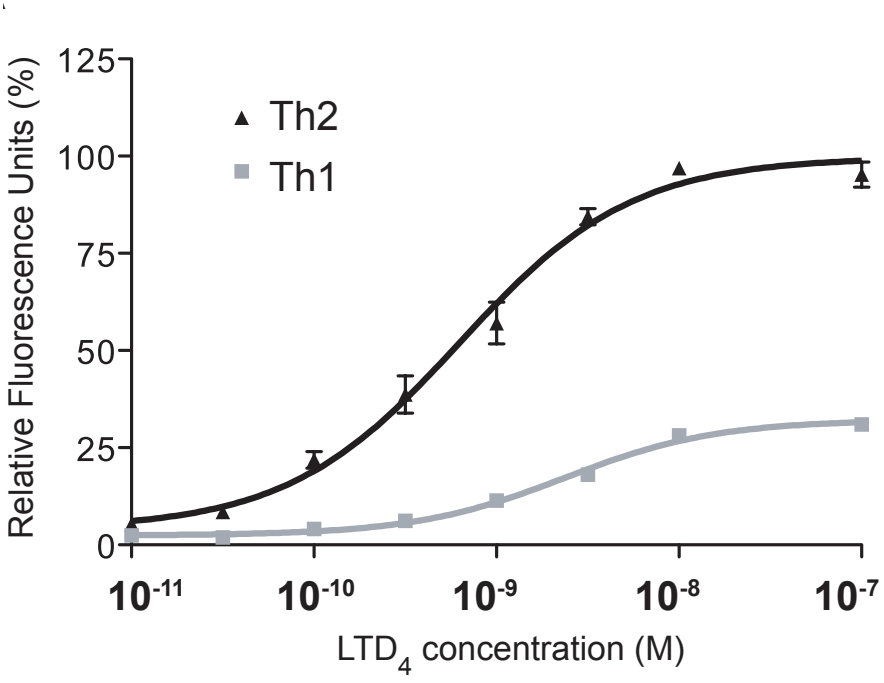


Figure 3.13 Th2 cells express functional CysLTR1 at higher levels than Th1 cells. Calcium flux in *in vitro*-differentiated Th1 and Th2 cells in response to increasing concentrations of LTD_4. A stronger calcium response is shown in Th2 cells compared to Th1 cells. Data are presented as mean ± SEM percentage of maximum response to LTD_4 from 3 experiments with different donors.

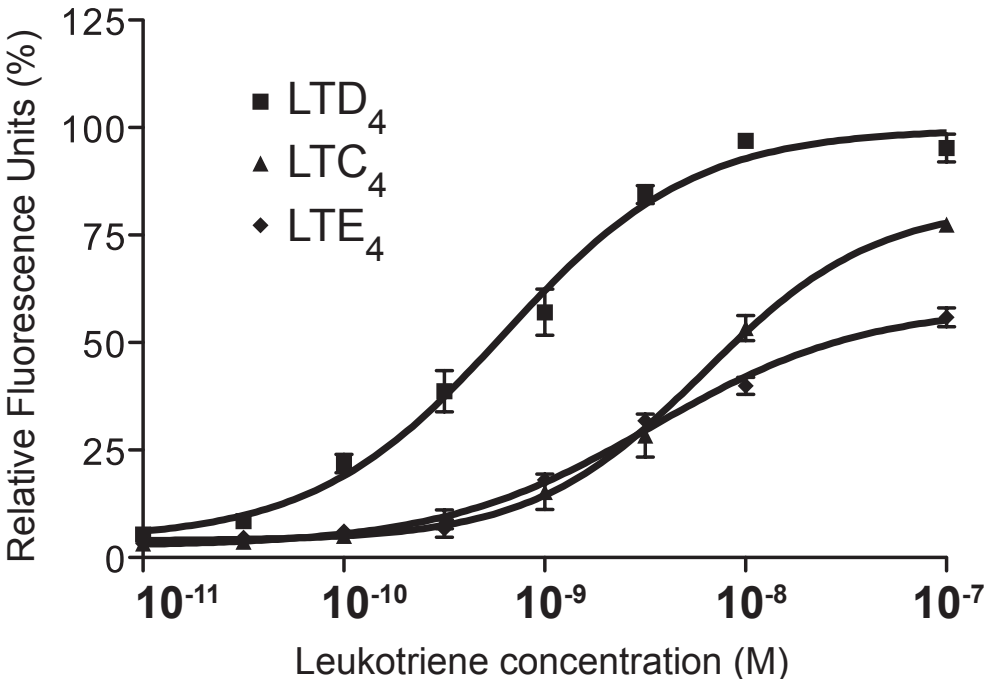


Figure 3.14 Th2 cells express functional CysLTR1 in LTD_4>LTC_4>LTE_4 rank order of potency. Calcium flux in Th2 cells in response to increasing concentrations of all three cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4, showing rank of potency as LTD_4>LTC_4>LTE_4. Data are presented as mean ± SEM percentage of maximum response to LTD_4 from 3 experiments with different donors.

3.2.4 Ca^{2+} flux via CysLT1R is inhibited by known CysLT1R antagonists

To confirm the identity of the cysteinyl leukotriene-responsive receptor expressed by Th2 cells and inhibit the calcium response, the selective antagonists MK571 (100 nM) and marketed antagonists Montelukast (10 nM) and Zafirlukast (10 nM) were used to treat Th2 cells prior to treatment with 100 nM LTD4. Calcium flux previously observed in Th2 cells was almost completely inhibited when using any of the three known $CysLT_1$ receptor antagonists (**Figure 3.15**). The results confirmed that the calcium flux in response to LTD_4 by human Th2 cells is dependent upon selective expression of functional CysLTR1.

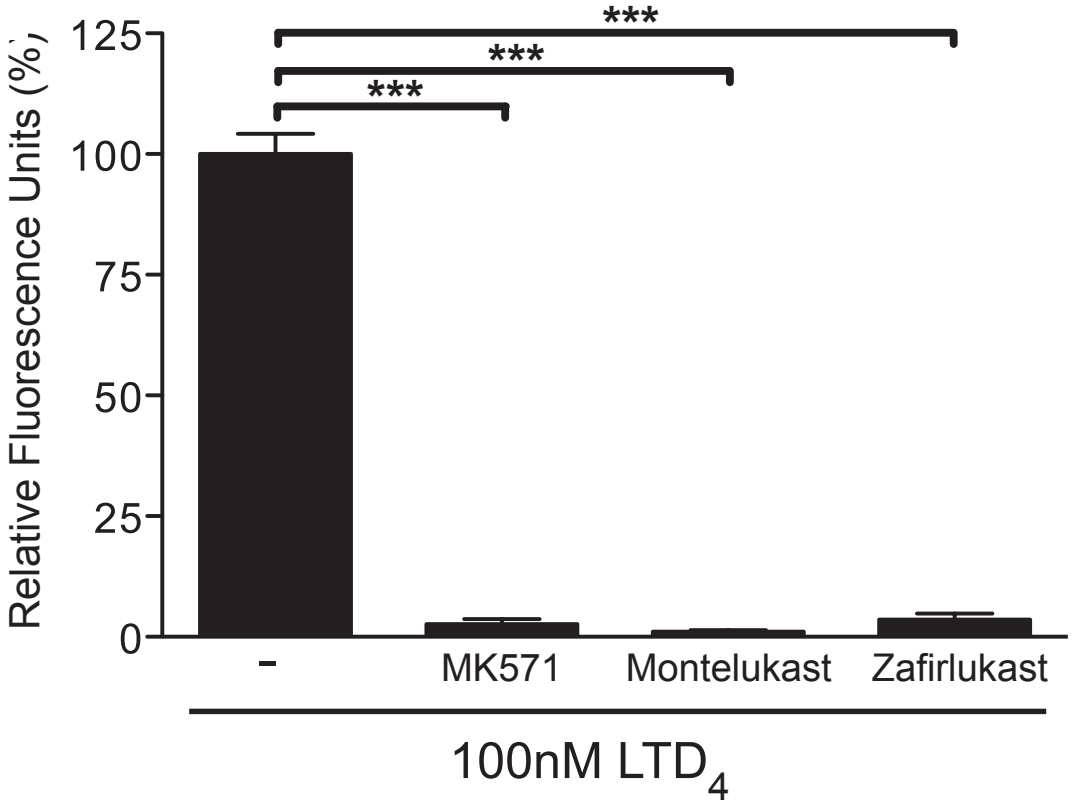
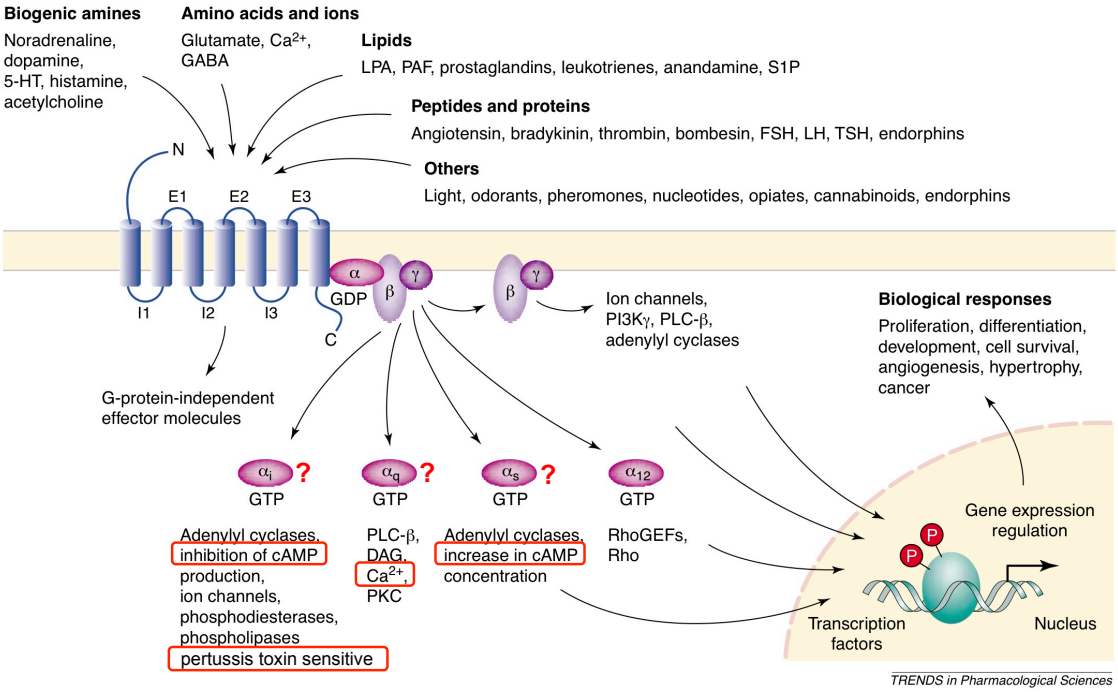


Figure 3.15 Inhibition of CysLTR1 functional expression in Th2 cells. Complete inhibition of calcium flux in Th2 cells in response to 100nM LTD_4 by 3 different cysteinyl leukotrienes antagonists MK571, montelukast and zafirlukast (100 nM and 10 nM). Data are presented as mean ± SEM percentage of maximum response to LTD_4 from 3 experiments with different donors. ***P < 0.001, 1-way ANOVA with Tukey post test.

3.2.5 CysLT1R is partially coupled to Gαi/o

From previous calcium signalling experiments, we found that intracellular calcium mobilization occurred in response to various doses of LTD_4 binding to the receptor, suggesting signalling via a Gαq pathway. To further characterise the signalling mechanisms involved in CysLT-induced calcium flux, we used the FLIPR method to investigate which G protein CysLT1R was coupled to (Schematic **figure 3.16**). Th2 cells were pre-incubated overnight with the Gαi/o inhibitor pertussis toxin and treated with increasing concentrations of LTD_4 (**Figure 3.17**). We found that, compared to Th2 cells with no pertussis toxin, pre-incubated Th2 cells showed a partial inhibition of intracellular calcium release of about 50-60% at the highest LTD_4 concentration since signalling was not completely inhibited. This suggested that $CysLT_1$ receptor is partially coupled to both Gαi and Gαq subunits.



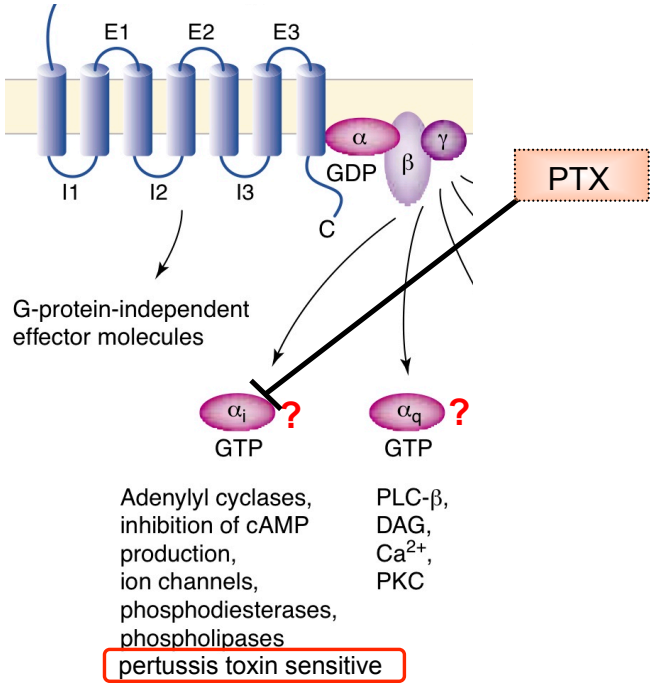


Figure 3.16 GPCR signalling via G protein. G protein-coupled signalling can occur via Gαi/o, Gαs, Gαq/11 or G12/13 and this can be confirmed by testing for cAMP, pertussis toxin sensitivity and calcium flux. Pertussis toxin can be used to determine whether the signalling is occurring via Gαi/o. Figures taken and adapted from (Marinissen and Gutkind 2001).

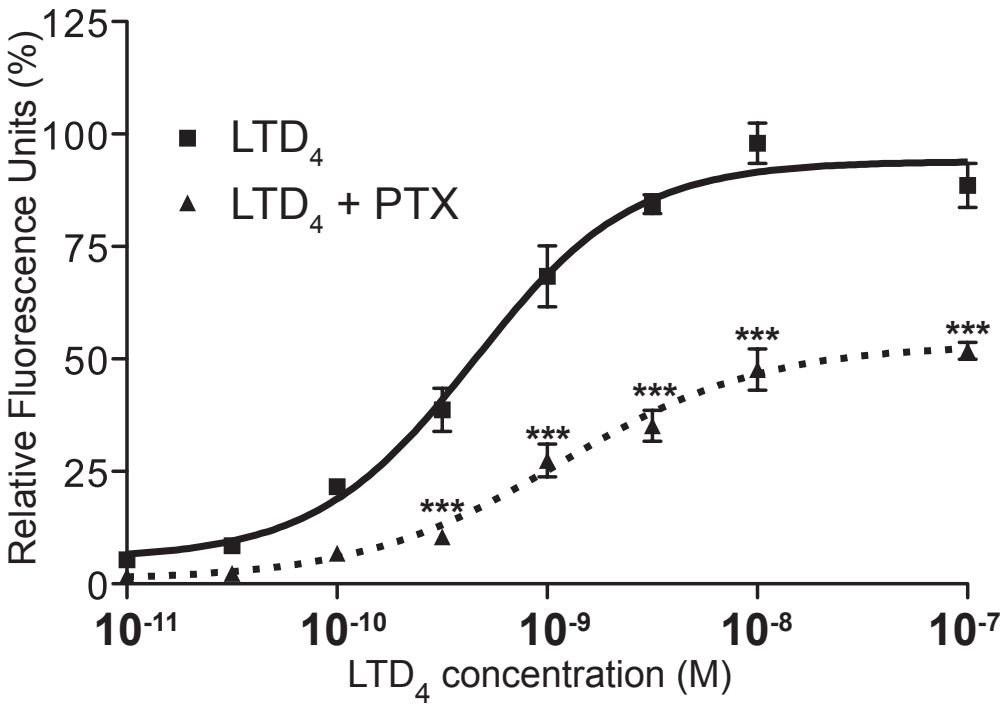


Figure 3.17 *Pertussis* toxin (PTX) partially inhibits calcium flux. The calcium response to LTD_4 in Th2 cells was inhibited by 50% when Th2 cells were incubated overnight with PTX. ***$P < 0.001$, 2-way ANOVA with Bonferroni post test.

3.2.6 CysLT1R is partially coupled to Gαq/11

To confirm the results obtained previously and the role of Gαi in CysLTR1 signalling, we looked at the cyclic AMP pathway using the competitive immunoassay HitHunter cAMP XS+ Assay (DiscoveRx). The DiscoveRx EFC (Enzyme Fragment Complementation, DiscoveRx technology) technology is based on two fragments of E. coli ß-galactosidase (ß-gal), a large protein fragment (enzyme acceptor, EA) and a small peptide fragment (enzyme donor, ED). Separately these fragments are inactive, but in solution they rapidly complement and recombine to form active ß-gal enzyme, which can hydrolyse substrate to produce luminescence. In this assay, free cAMP from cell lysates compete for antibody binding against labelled cAMP (ED-cAMP conjugate is free to complement EA to form active enzyme by EFC, which subsequently hydrolyses substrate to produce a luminescent signal. A positive signal generated is directly proportional to the amount of free cAMP bound by the binding protein (as seen in **figure 3.18**, www.discoverx.com).

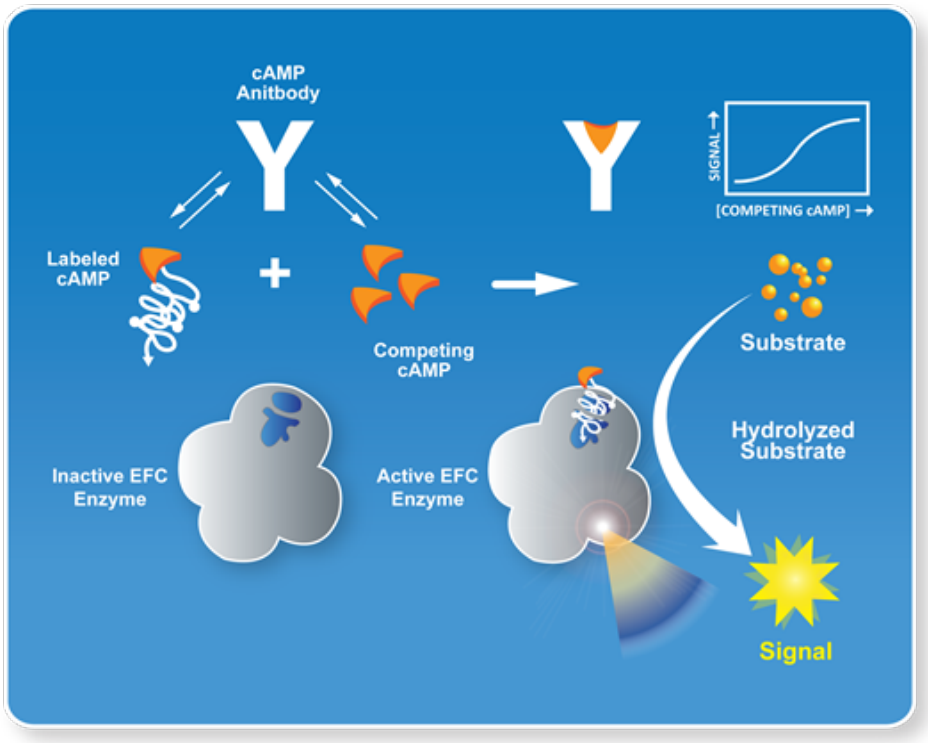


Figure 3.18 HitHunter EFC Assay Principle. In the assay, ED is conjugated to the ligand of interest (cAMP) which competes against free ligand for binding to a binding protein. Depending on the ligand of interest, the binding protein can be an antibody, an enzyme, or a receptor. Taken from www.discoverx.com (DiscoveRx).

As previously mentioned, signalling via the Gαi/o subunit is also characterised by a decrease in intracellular cyclic AMP. In this experiment, we examined whether LTD_4 could inhibit cAMP signalling in Th2 cells.

In order to optimise the assay, we first tested cysteinyl leukotrienes alone to check whether there was a measurable cAMP signal or if we should amplify the signal using forskolin. Forskolin is known to resensitize cell receptors by activating the enzyme adenylyl cyclase and increasing the intracellular levels of cAMP. As seen in **Figure 3.19**, none of the cysteinyl leukotrienes LTD_4, LTC_4, LTE_4 induced any change in cyclic AMP, even at 100 nM, compared to the signal detected by 100nM forskolin alone.

Following the results in **figure 3.19**, we decided to use forskolin to induce cAMP before adding LTD_4, LTC_4 and LTE_4 to check the effect of cysteinyl leukotrienes on cyclic AMP. Initially we optimised the cyclic AMP assay testing various doses of forskolin alone and decided that 20 µM forskolin would induce a robust increase in cAMP levels and appeared to be the optimal dose to use for the assay. We proceeded to use 20 µM forskolin alone at various doses, compared in both Th1 and Th2 cells (**Figure 3.20**). **Figure 3.20** shows a dose response for forskolin alone in Th1 and Th2 cells. We decided to use a concentration between 100 µM and 10 µM and followed on our experiments using 20 µM.

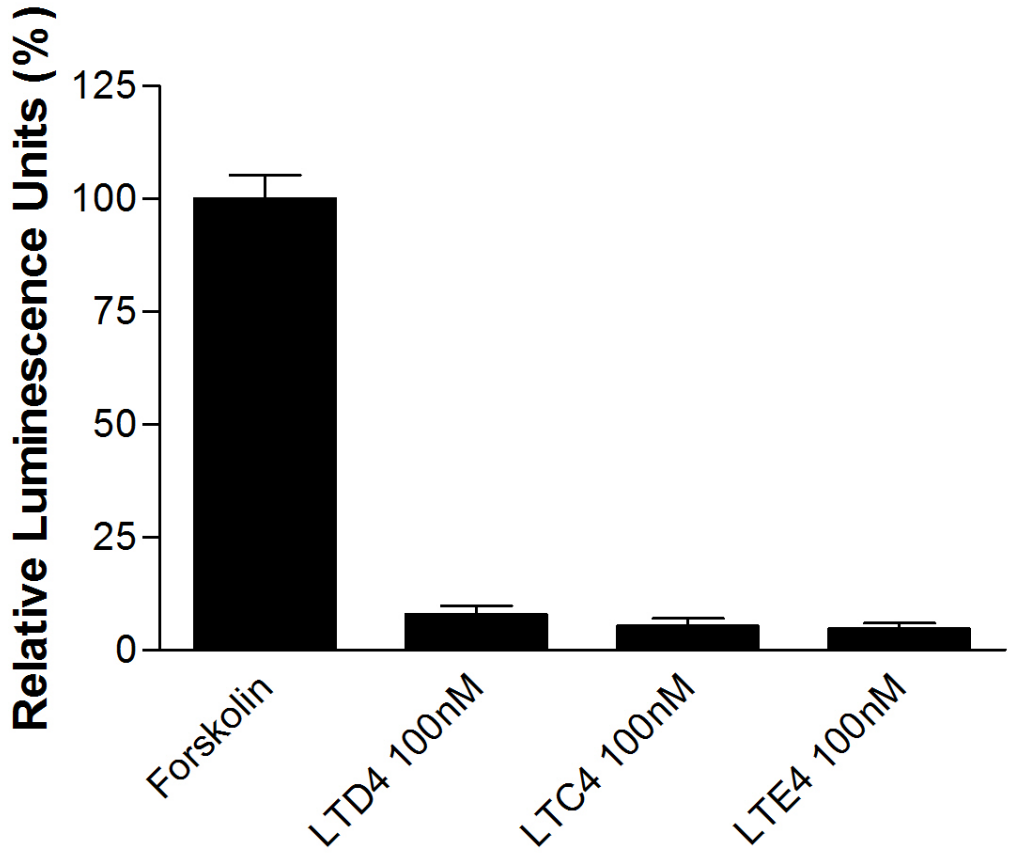


Figure 3.19 cAMP detection in Th2 cells using LTD_4, LTC_4 and LTE_4. cAMP was measure when using 100nM forskolin alone, compared to 100 nM LTD_4, 100 nM LTC_4 and 100 nM LTE_4 alone. LTD_4, LTC_4 and LTE_4 did not induce cyclic AMP on their own. Data are representative of 3 biological replicates.

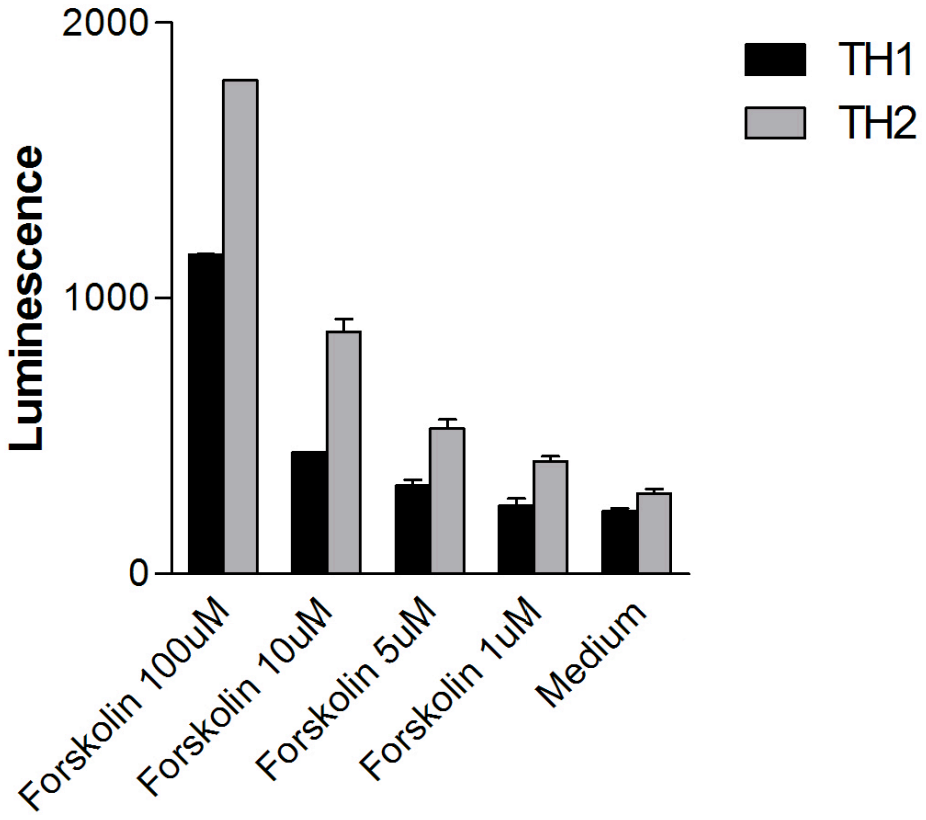


Figure 3.20 Comparison of cAMP detection in Th1 and Th2 cells. Dose response for forskolin using various doses of forskolin alone, S1P alone and forskolin with S1P. Data are representative of 3 biological replicates.

The optimum results were seen when using 20nM forskolin and we therefore proceeded with additional experiments. We went on to test some known positive controls such as S1P and PGD2, as seen in **figures 3.21** and **3.22**, respectively. S1P has been shown to induce cAMP via its S1P receptors lymphocytes in smooth muscle cell (Damirin et al. 2005; Spiegel and Milstien 2003b, 2003a), and its receptor has been shown play an important role in lymphocyte entry, via G protein signaling (Graler 2010; Lo et al. 2005)**.** As expected, S1P inhibited cAMP levels by 50% in Th2 cells. Interestingly, PGD2 only inhibited cAMP by approximately 10%.

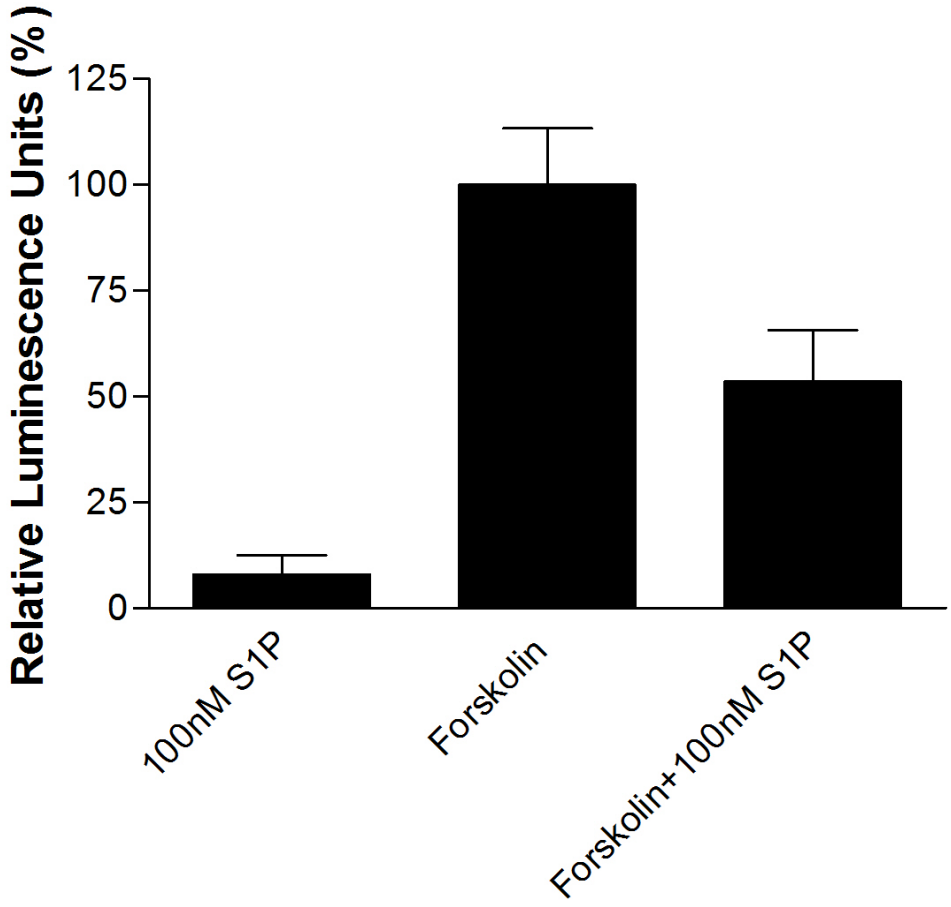


Figure 3.21 S1P-mediated 50% inhibition of cAMP signaling in Th2 cells. Cells were treated with 20nM forskolin to induce cAMP, and S1P. All data are expressed as mean ± SEM percentages of maximum response to S1P or forskolin from 3 experiments with different donors.

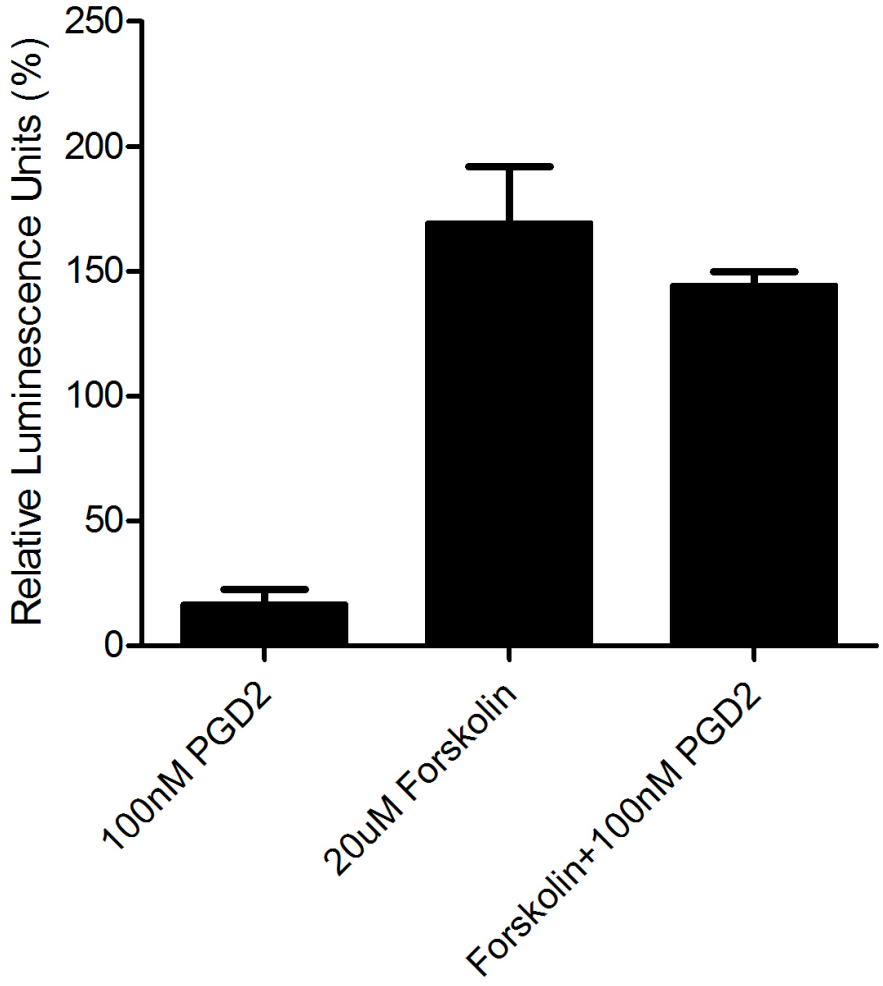


Figure 3.22 PGD2 does not inhibit cAMP signaling in Th2 cells. Cells were treated with 20nM forskolin to induce cAMP, and 100nM PGD2. All data are expressed as mean ± SEM percentages of maximum response to S1P or forskolin from 3 experiments with different donors.

Following the testing of known positive controls S1P and PGD2, we continued using 20 nM forskolin but this time with various doses of LTD_4 using Th2 cells as shown in figure 14. As seen in previous experiments, 100 nM LTD_4 alone did not induce cyclic AMP. Adding various doses of LTD_4 to 20 nM forskolin resulted in a concentration-dependent decrease in cyclic AMP signalling. Adding 0.1nM and 1nM LTD_4 to Th2 cells showed a 25% decrease in cyclic AMP, 10 nM LTD_4 showed a 45% decrease and 100 nM showed a 50% decrease in cyclic AMP (**Figure 3.23**).

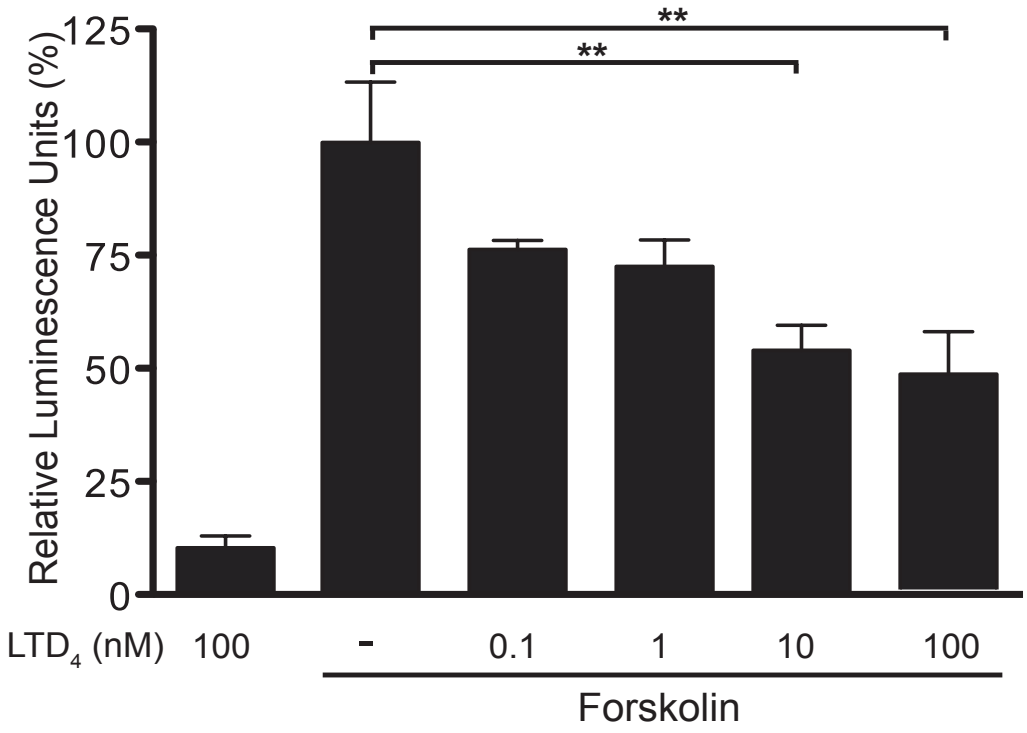


Figure 3.23 LTD_4-mediated inhibition of cAMP signaling in Th2 cells. Cells were treated with forskolin to induce cAMP, and increasing concentrations of LTD4 inhibited cAMP generation. All data are expressed as mean ± SEM percentages of maximum response to LTD_4 or forskolin from 3 experiments with different donors. $^{**}P < 0.01$, 1-way ANOVA with Tukey post test.

Additional experiments using $CysLT_1$ receptor antagonists showed that pre-treatment of the Th2 cells with the $CysLT_1R$ selective antagonist MK571 completely blocked the LTD_4 dose-dependent inhibition of cAMP signalling (**Figure 3.24**), confirming that the receptor is partially coupled to Gαq/11 and Gαi/o, corresponding to our previous data and data in the literature and confirming that the LTD_4-induced decrease in cyclic AMP is due to CYSLTR1-mediated effects.

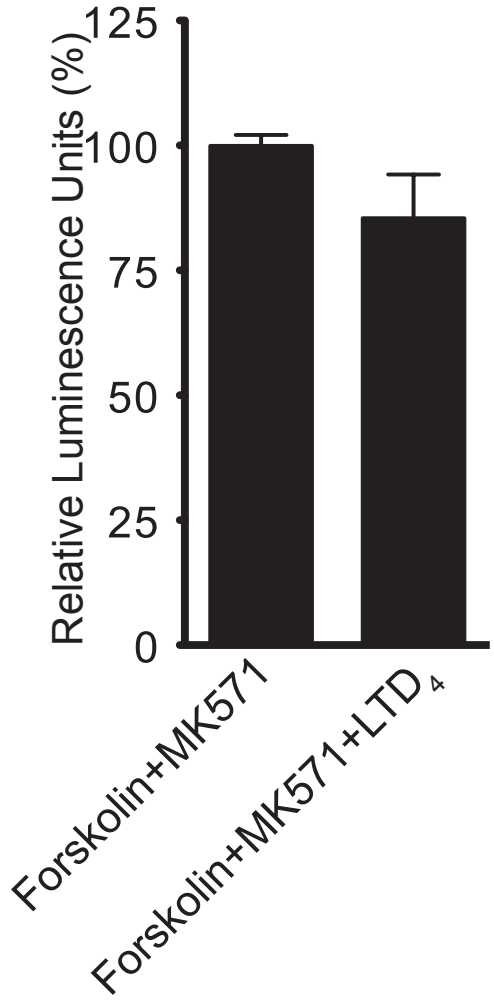


Figure 3.24 Blocking of the LTD_4-mediated inhibition of cAMP response by MK571. 100nM LTD_4 was used in these experiments. All data are expressed as mean ± SEM percentages of maximum response to LTD_4 or forskolin from 3 experiments with different donors.

Similar experiments were performed using a range of LTC_4 and LTE_4 doses, as seen in **figure 3.25** and **figure 3.26**. Interestingly when using LTC_4, the levels of cyclic AMP increased in a concentration-dependent manner**,** instead of decreasing, as seen with LTD_4. 0.1nM LTC4 induced a 70% reduction in cAMP, 1nM a 65% reduction, 10nM 45% and 100nM 50%**.** The dose response observed when using LTE_4 was similar to results seen using LTD_4, with a maximum reduction of 60% in cyclic AMP levels when using 100 nM and 1 nM LTE_4, 40% reduction with 10nM LTE_4 and 30% reduction when using 0.1 nM LTE_4.

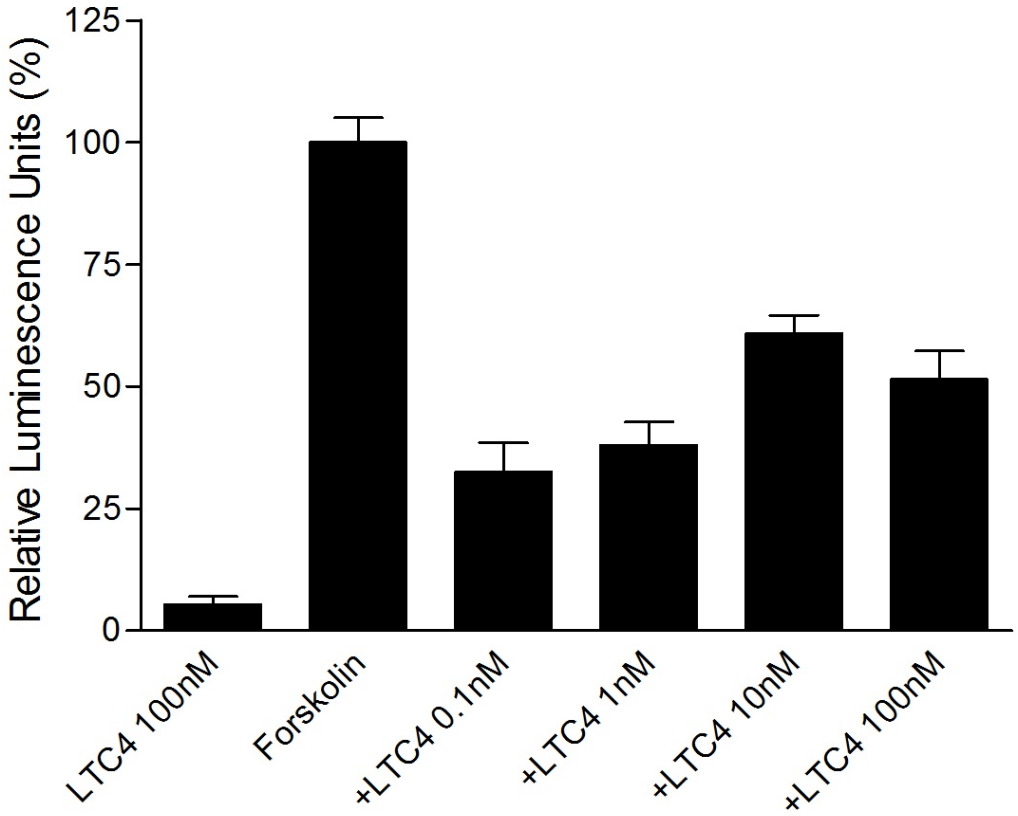


Figure 3.25 LTC_4-mediated inhibition of cAMP signaling in Th2 cells. Cells were treated with 20nM forskolin to induce cAMP, and increasing concentrations of LTC_4 decreased the cAMP generation. All data are expressed as mean ± SEM percentages of maximum response to LTC_4 or forskolin from 3 experiments with different donors.

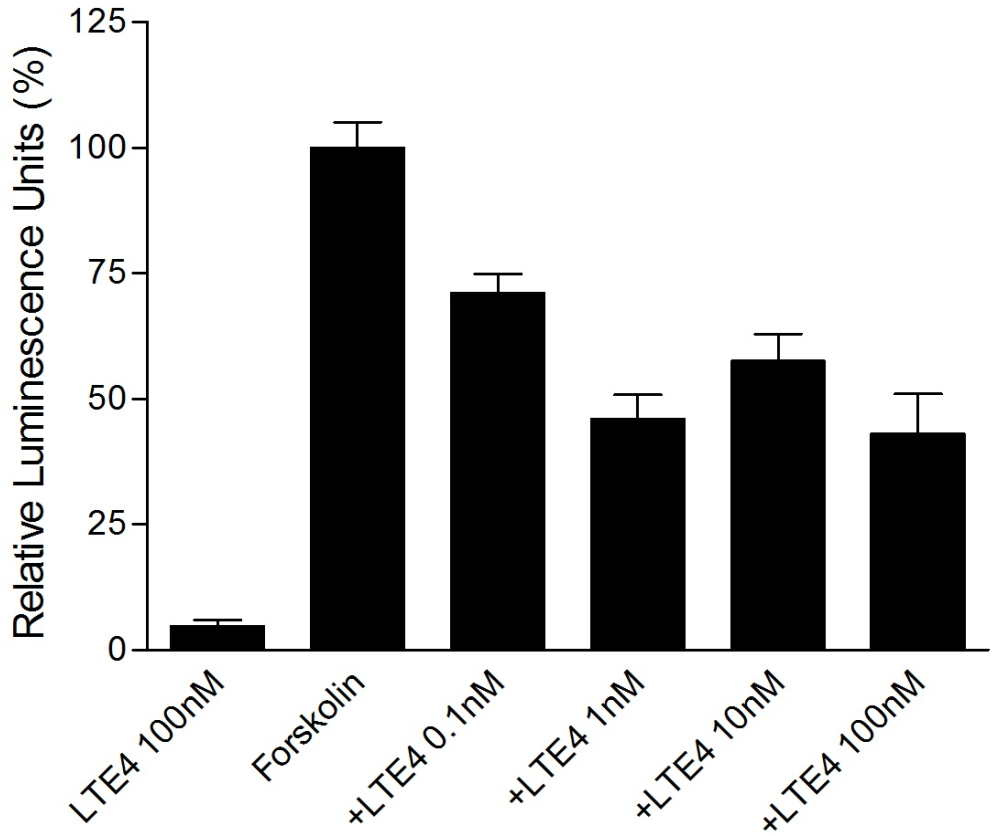


Figure 3.26 LTE_4-mediated inhibition of cAMP signaling in Th2 cells. Cells were treated with 20 nM forskolin to induce cAMP, and increasing concentrations of LTE_4 increased the cAMP generation similarly to LTD_4. All data are expressed as mean ± SEM percentages of maximum response to LTE_4 or forskolin from 3 experiments with different donors.

Following the cyclic AMP experiments, we decided to investigate the origin of calcium observed in the previous calcium assays.

3.2.7 Calcium signalling in Th2 cells

Additionally, we identified the source of calcium measured in the calcium assays. Pre-incubation of Th2 cells with thapsigargin to deplete calcium from intracellular endoplasmic reticular calcium stores completely inhibited calcium mobilization in response to LTD_4. Thapsigargin (THP) is a non-competitive SERCA inhibitor, which blocks the calcium pump on the surface of the endoplasmic reticulum. By blocking the receptors, it stops calcium being pumped back into the sarcoplasmic and endoplasmic reticulum, which raises cytosolic calcium levels and eventually depletes intracellular calcium stores. Store depletion as we performed generally induces secondary activation of plasma membrane calcium channels, particularly calcium-release activated calcium channels (or CRAC channels), whereby the cell sends signals to the cell surface calcium channels to be opened and allow an influx of calcium into the cytosol. To confirm this occurrence in our system, we used EDTA to block extracellular calcium which may be pumped into the cell following intracellular store depletion. Chelation of extracellular calcium by pre-incubation of Th2 cells with EDTA partially inhibited calcium flux by approximately 40-50% (**Figure 3.27**). This data suggests that LTD_4 signalling through CysLTR1 activates store-operated calcium channels in Th2 cells, with the primary source of calcium originating from intracellular stores, and the secondary source extracellular calcium being pumped into the cell.

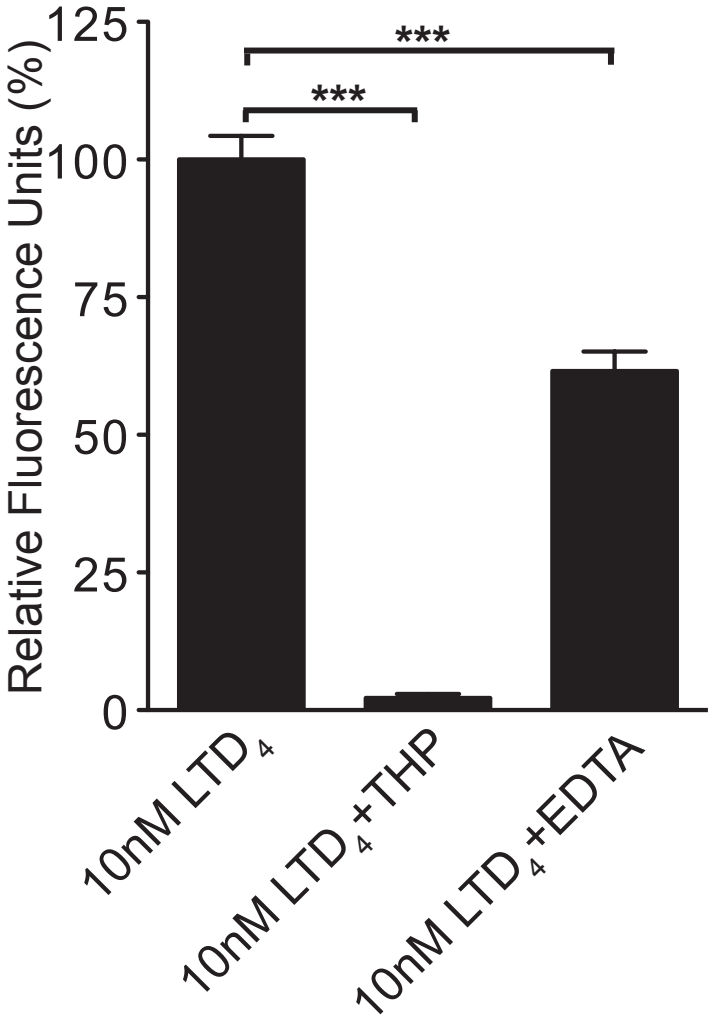


Figure 3.27 Source of calcium in LTD_4-driven calcium flux. Thapsigargin (THP)-mediated inhibition and EDTA-mediated partial inhibition of calcium flux responses to LTD_4 in Th2 cells. Data are expressed as mean ± SEM percentages of maximum response to LTD_4 from 3 experiments with different donors. ***P < 0.001, 1-way ANOVA with Tukey post test.

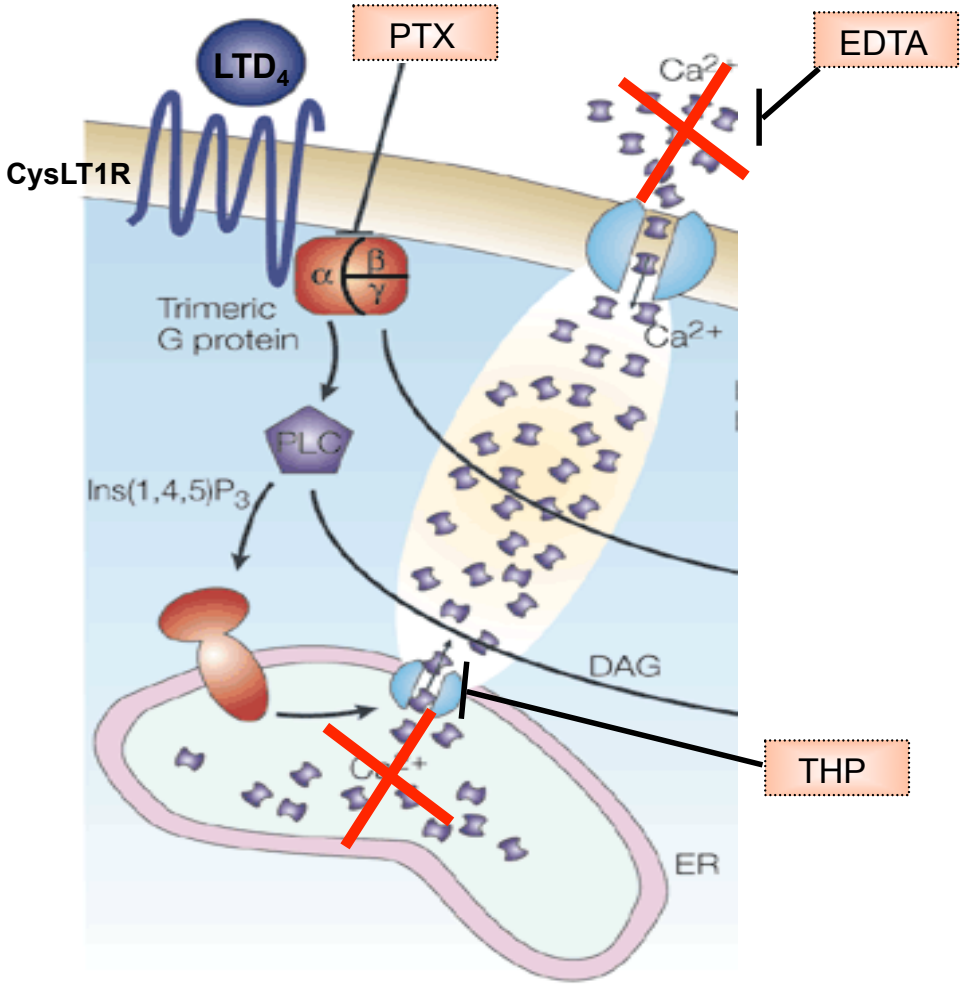


Figure 3.28 Representation of intracellular calcium stores. The origin of calcium can be tested by using the extracellular calcium-chelating agent EDTA and/or THP (thapsigargin) to deplete endoplasmic reticular calcium stores.

3.2.8 Summary

Using in-vitro differentiated human Th1 and Th2 cells, we have demonstrated that CYSLTR1 is expressed on both Th1 and Th2 cells. LTD_4, the known physiological ligand with highest affinity for CysLT1R, caused calcium mobilisation in both Th1 and Th2 cells with much higher levels of calcium flux in Th2 cells. The other two known ligands LTC_4 and LTE_4 also caused calcium signalling in Th2 cells and the response was dose-dependent and ligand affinity-dependent. The calcium response was completely inhibited by known $CysLT_1$ receptor antagonists MK571, Montelukast and Zafirlukast.

In these experiments, we have shown that the human CysLT1R is coupled to both Gαi/o and Gαq/11 in our *in vitro*-differentiated human Th2 cells system. This corresponds to previous findings that the receptor is partially coupled in native systems but only coupled to Gαq/11 in recombinant systems. We showed this via two different measurements: one measured calcium flux following pertussis toxin incubation, the other measured cyclic AMP levels after adding LTD_4. This confirms that CysLTR1 is pertussis toxin-sensitive in Th2 cells. Furthermore, we have shown that the calcium flux measured in our experiments is sourced in the endoplasmic reticulum calcium stores and that, following depletion of these stores, the cell signals to open membrane calcium channels to allow influx of extracellular calcium into the cytosol.

Although other studies have reported the expression of CYSLTR1 in various types of cells, this data consist of the first report that CYSLTR1 is expressed in human T cells. We show for the first time that human Th2 cells selectively express CYSLTR1 mRNA with 6.5 fold higher expression than human Th1 cells (**Figure 1C**). We also found no

evidence for expression of the other known CysLT receptors, CYSLTR2, GPR17 or P2YR12 in T cells.

During the course of these studies other evidence emerged supporting out finding that T cells may be a CysLT target cell type. The first evidence we have found was a study by Chtanova *et al.* where transcriptional profiling of human T-cell subsets was performed (Chtanova et al. 2004). They investigated the function, identity and molecular interactions of another subset of human effector T cells, T follicular helper cells (Tfh). For this, CD57+CXCR5+CD4+ and CD57-CXCR5+CD4+ T cells were isolated from human tonsils and assessed by Affymetrix microarrays for gene expression in a similar manner to our study. Alongside T_{FH}, they also made a comparison between Tfh cells and Th1 and Th2 cells, as well as comparing to T_{EM} (effector memory) and T_{CM} (central memory) cells isolated from peripheral blood. In their analysis CysLT1R was listed as being upregulated in human Th2 cells. Unfortunately no additional comments were made on this finding and no further investigations were performed to examine CysLT responsiveness.

Prinz *et al*. found further evidence for a role Cysltr1 in T-cells in a study examining mice with mutations in the adaptor molecule linker for activation of T cells (LAT), *LatY136F* αβ and *Lat3YF* γδ T cells expressed high levels of Cysltr1. Binding of LTD_4 to the receptor caused Ca^{2+} flux and chemotaxis in both *LatY136F* αβ and *Lat3YF* γδ T cells. Wild-type *in vitro*-activated mice T cells, but not resting T cells, also migrated toward LTD_4 however with a lower magnitude than T cells freshly isolated from *LatY136F* and *Lat3YF* mice. These mice with mutations of distinct LAT tyrosine residues (*LatY136F* and *Lat3YF*) develop lymphoproliferative disorders involving TCR αβ or γδ T cells that

trigger symptoms resembling allergic inflammation. *LatY136F* (mutation of tyrosine 136 in LAT into phenylalanine) caused a fatal lymphoproliferative disorder involving polyclonal CD4+ T cells that chronically produced type 2 cytokines such as IL-4, IL-5, and IL-13. *Lat3YF* (mutation in tyrosine 175, 195 and 235 into phenylalanine) resulted in the selective development and expansion of γδ T cells, which spontaneously deployed a Th2-like effector program. Both mutated cell types behaved similarly to effector T cells, particularly following activation with ionomycin and their subsequent activation of effector functions and ability to produce Th2-specific cytokines. Given that the T-cells they were investigating appear to have Th2-like properties this study strongly supports our findings that Th2 cells respond to CysLTs even though they were examining mutant murine T cells (Prinz et al. 2005).

One final piece of evidence for Cysltr1 expression on T cells was found in a study using knock-in mice expressing a GFP-Foxp3 fusion protein, in which a population of CD4+CD25+Foxp3- T cells were identified as effector T cells. Transcriptional profiling of these cells revealed Cysltr1 expression, which was not present in naïve CD4+CD25-Foxp3- T cells and CD4+CD25+Foxp3+ regulatory T cells (Fontenot et al. 2005).

Collectively our data and the literature strongly suggest that T cells, in particular Th2 cells, should be considered a legitimate target cell type for CysLTs. The next chapter will examine the functional effects that CysLTs have on human Th2 cells.

CHAPTER 4
RESULTS: FUNCTIONAL EFFECT OF LTD4 ON TH2 CELLS

Chapter 4 Results – Functional effect of LTD_4 on Th2 cells

4.1 Introduction

4.1.1 Characterisation of CysLTR1 functions in human T cells

In the previous chapter we demonstrated that human Th2 cells selectively express CYSLTR1 mRNA and that this results in functional expression of the receptor as evidenced by responsiveness to CysLTs via calcium mobilisation. Additional experiments indicated that the CysLT1R receptor was partially coupling via Gαi and Gαq and these results were confirmed both via calcium assays using pertussis toxin and cyclic AMP assays. In all assays, the known $CysLT_1$ receptor antagonists inhibited the LTD_4 response. Finally, we also reported that calcium appeared to originate primarily from intracellular calcium stores. To examine the functional consequences of CysLTR1 signalling in Th2 cells we wished to study several facets of T cell biology, specifically migration (chemotaxis), cytokine expression and gene transcription. These studies were to some extent guided by the known functions of CysLTs *in vivo* and in other cell types.

4.1.2 CysLTR1 involvement in chemotaxis

G protein-coupled receptors (GPCRs) have been widely studied in chemotaxis assays since many of them are the key receptors to which leukotrienes, chemokines and other mediators bind to in order to effect cellular migration. GPCRs are found on the surface of target cells and interact with mediators typically of nanomolar affinity to subsequently transduce intracellular signalling events. Immunological processes such as antigen recognition, lymphocyte priming and effector responses to inflammation are executed by leukocytes and their ability to circulate around and migrate through

body tissues is critical for these functions. Leukocyte trafficking is regulated by a complex interaction of molecules, such as leukotrienes, cytokines and chemokines, all with roles in the chemotactic response. Chemotaxis can be defined as a directed movement of a cell towards a chemical signal or chemotactic stimuli, in the case of leukocytes usually mediated via cell surface receptors.

The major role of these mediators such as leukotrienes and chemokines is to guide cells for migration by providing them with directional cues. Migrating cells follow a signal of increasing mediator concentration towards the source of the mediator, very often to migrate to a site of inflammation (D'Ambrosio et al. 2001).

LTD_4 has been reported as being able to induce chemotactic responses from several cell types, including eosinophils, neutrophils, basophils, monocytes and haematopoietic stem cells. One study used the chemotaxis under-agarose technique to demonstrate that LTD_4 was a potent chemoattractant for eosinophils at various doses and this migration was inhibited using the SK&F 104353 antagonist (Spada et al. 1994). However, LTD_4 had little effect on the eosinophil chemokinesis. LTB_4 also induced chemotaxis of eosinophils. Both LTB_4 and LTD_4 induced chemotaxis and chemokinesis in neutrophils (Krauss et al. 1994; Spada et al. 1994).

In a randomized controlled trial, another study reported that four weeks of Montelukast treatment reduced airway eosinophilic inflammation in asthmatic subjects suggesting an effect on eosinophil migration (Pizzichini et al. 1999). Following reports that LTE_4 can increase numbers of eosinophils (10-fold higher than neutrophils) and neutrophils in the human airway mucosa 4 hours after inhalation in subjects (Laitinen et al. 1993), additional studies found that LTD_4 also induced eosinophilia in

sputum of asthmatic subjects 4 hours after inhalation (Diamant et al. 1997). Interestingly, reports indicated no significant change in numbers of lymphocytes, plasma cells, mast cells or macrophages in response to LTD_4 (Laitinen et al. 1993). It was also shown that eosinophils isolated from non- atopic venous blood migrated to LTD_4, and this was inhibited using pranlukast in a dose-dependent fashion (Ohshima et al. 2002). This was also reproduced by another group, who demonstrated using the Boyden chamber system that human eosinophils migrated to LTD_4, LTC_4 and LTE_4 and this was inhibited by Montelukast (Fregonese et al. 2002). Conversely, it was also reported in a comparison between inhaled LTD_4 and LTE_4 that LTE_4 causes an increase in sputum eosinophils but LTD_4 did not (Gauvreau et al. 2001).

In guinea pig tracheal explant preparations, it was found that both LTB_4 and LTD_4 facilitated eosinophil migration from the lamina propria to the lumen caused by formyl-met-leu-phe (FMLP) (Munoz et al. 1997). Using radio-labelled guinea-pig conjuctiva eosinophils injected into recipient animals, Chan *et al.* found that LTD_4 and PAF induced a significant 2.5 fold increase in chemotaxis *in vivo*, which was inhibited using MK571, a known $CysLT_1$ receptor antagonist (Chan et al. 1990). Similarly, inhalation of aerosolized LTD_4 elicited eosinophil migration into guinea pig lungs, which was maintained up to 4 weeks post challenge (Underwood et al. 1996) again implicating LTD_4 in the migration of eosinophils.

In 2005 basophils were analysed for their expression and function of CYSLTR1, (Gauvreau et al. 2005) following previous studies by the group reporting basophil accumulation in asthmatic airways after inhalation of LTE_4 (Gauvreau et al. 2001).

Although it seems to report migration of basophils to LTD_4, the assays were problematic and the evidence for the chemotaxis of basophils is unclear.

Cysteinyl leukotrienes have also been shown to be chemotactic to hematopoietic progenitor cells. CD34+ hematopoietic progenitor cells isolated from peripheral blood mononuclear cells from normal patients were found to express CYSLTR1 and also to migrate to LTD_4 (Bautz et al. 2001). RT-PCR was used to examine the expression of CYSLTR1 mRNA in CD34+ cells, mature leukocytes and in monocytic cell line THP-1 used as a positive control, CD34+ hematopoietic cell line KG1a and CD34- myeloid cell line HL-60. Calcium experiments revealed that LTD_4 induced a significant increase in intracellular calcium in peripheral blood CD34+ cells. Incubation with pertussis toxin resulted in partial inhibition of the calcium response. Using a modified Boyden chamber system for the chemotaxis assay, LTD_4 was seen to cause migration of peripheral blood CD34+ cells at various concentrations (Bautz et al. 2001).

In a study described in the previous chapter, using $\alpha\beta$ and $\gamma\delta$ T cells from mice with knock-in mutations (*Lat3YF* and *LatY136F*, respectively) strongly migrated to LTD_4. Wild-type comparison revealed that *in vitro*-stimulated CD4+ T cells also migrated to LTD_4 but with a lower magnitude and unstimulated CD4+ T cells did not migrate towards LTD_4 at all. Interestingly these mutant cells caused a fatal lymphoproliferative disorder involving polyclonal CD4+ T cells that chronically produced type 2 cytokines such as IL-4, IL-5 and IL-13 and which spontaneously deployed a Th2-like effector program, characterized by lymphocytic infiltration of the lungs and tissue eosinophilia, symptoms reminiscent of allergic inflammation. This latter study is the only report of

chemotaxis observed in CD4+ T cells, however these were from *LatY136F* and *Lat3YF* mutant mice that had LAT-mutated T cells (Prinz et al. 2005).

Several studies have investigated the role of CYSLTR1 expression in monocytes. Thivierge *et al*. investigated the potential for cytokines such as IL-13 and IL-4 to modulate the expression and function of CysLT1R. The group found that CysLT1R mRNA was constitutively expressed by human monocytes and that treatment of cells with IL-13 augmented the CysLT1R mRNA levels by 3- to 5-fold. This increase was time-dependent and observed as early as 4 hours post stimulation, with a maximal response at 8 hours and sustained for 24 hours. Similar results were observed by treating the cells with IL-4, but not IFN-y. In both cases, protein levels were also increased. Thivierge *et al*. investigated the effect of IL-4 and IL-13 on calcium mobilization and the functional responsiveness of the cells to LTD_4. Treatment of monocytes with IL-13 or IL-4 led to an increased calcium flux in response to LTD_4 but not LTB_4. Treatment of monocyte-derived macrophages led to an even larger increase than in monocytes. These responses were fully inhibited by antagonist MK571. Thivierge *et al.* also investigated the effect of IL-13 and IL-4 on the migration of monocytes towards LTD_4. Resting monocytes did not show much chemotactic response to LTD_4. However monocytes that had been incubated with IL-13 or IL-4 showed a significant increase in chemotaxis (Thivierge et al. 2001). In a further study by Thivierge (Thivierge et al. 2006), monocyte-derived dendritic cells stimulated and matured with LPS (which binds TLR4) showed a near total loss of their CysLTR1 expression. This was also observed when stimulating the cells with zymosan, which is known to bind to TLR2. Interestingly, results showed that maturation with polyI:C had no effect on CYSLTR1 expression.

Inhibition of LPS-induced COX-2 by COX-2 inhibitor NS-398 reversed the downregulation of CysLTR1 in LPS-stimulated DCs. PGE2 and TNF-α combined also reduced the CYSLTR1 expression to very low levels. These results were also observed at the mRNA level, which was decreased by 70% to 80% in LPS- and PGE2/TNF-α-matured DCs, respectively. In contrast to the MoDCs, human monocytes were resistant to CysLT1R downregulation and this suggested the differentiation process in DCs was the key to rendering the cells sensitive to LPS and PGE2/ TNF-α. Cells matured with LPS showed no calcium response to LTD_4 in contrast to polyI:C-matured DCs. The same effect was noted on chemotaxis (Thivierge et al. 2006).

Woszczek *et al*. showed that LTD_4 induced signalling, gene expression and migration of monocytes and monocyte-derived immature dendritic cells (iDCs) and that these effects could be inhibited by IL-10 (Woszczek et al. 2008a). They also found that CysLT2R and GPR17 were also expressed on iDCs. In their experiments, they cultured monocytes in the presence of IL-10 for up to 24 hours and measured mRNA levels of CYSLTR1 to find that IL-10 reduced mRNA expression in a dose-dependent manner, with an optimal result after 6 hours. Similar results were observed in iDCs, which were cultured without IL-4 prior to the experiment, as IL-4 is known to upregulate CYSLTR1 expression in these cells. In iDCs, the response was time-dependent. Calcium flux experiments revealed that LTD_4 induced a strong calcium response in both monocytes and iDCs and this was significantly inhibited when both types of cells were incubated overnight with IL-10. Additional tests revealed that IL-10 was also capable of inhibiting LTD_4-induced immediate-early genes FOSB and EGR2 in monocytes but also in iDCs, in a way similar to MK571. siRNA of the receptor confirmed that the results observed

previously were all CysLT1R-mediated, including intracellular calcium and gene expression. IL-10 also inhibited the migration of monocytes and iDCs towards LTD_4. In additional experiments, IL-10 was also found to enhance the LTD_4-induced chemotaxis of iDCs to CCL5 and CCL3 (Woszczek et al. 2008a). Monocytes were also found to chemotax to both LTB_4 and LTD_4 with an increase in migration towards both leukotrienes together (Chen et al. 2011).

Kato *et al.* showed that LTD_4 had an enhancing effect on the migration of normal human lung fibroblasts to human plasma fibronectin. Despite claiming that LTD_4 potentiated the chemotaxis, the results showed a one-fold increase in the migrated cells when LTD_4 was added to the normal lung fibroblasts before incubation (Kato et al. 2005).

A study by Espinosa *et al*. showed that exposure of bronchial smooth muscle cells (BSMCs) to TGF-β, IL-13 or IFN-γ increased the protein expression of CysLTR1 on these cells (Espinosa et al. 2003). Surprisingly, IL-4 had no effect on the protein expression of CysLT1R in human BSMCs. mRNA levels studies revealed that IL-13 and IFN-γ both increased mRNA levels but not TGF-β, and this after a long exposure of 24 hours. The study then investigated the effect of LTD_4 on the proliferation of the cells on both control cells and TGF-β, IL-13- and IFN-γ-stimulated cells. Results showed no significant proliferation in control BSMCs. However, a 24-hour stimulation with IL-13 or TGF-β induced a significant proliferation in BSMCs (Espinosa et al. 2003).

Since CysLTs, acting through $CysLT_1$ receptor, are chemotactic for several immune cell types we hypothesized that they may also be chemotactic for human Th2 cells. In our experiments, we investigated the chemotactic effect of LTD_4 on Th2 cells and this was

tested at various doses of LTD_4. We also tested known positive controls such as SDF-1α and PGD2. Additionally we examined whether chemotaxis could be inhibited by antagonist MK571.

In further experiments, we looked at the effect of LTD_4 on Th1 and Th2 cells proliferation, differentiation and cytokine expression.

4.2 Results

4.2.1 Human Th2 cells migrate to LTD4 in a dose-dependent manner

We next aimed at determining the functional consequence of CysLTR1 signaling on human Th2 cells. The mechanism of recruitment of T cells and other inflammatory cells to the lung during inflammation has been the focus of research for a long time and is of high interest since blocking T cell recruitment to the lung would be a potential therapeutic strategy for asthma and other inflammatory lung diseases.

Cysteinyl leukotrienes have previously been shown to be chemotactic agents for haematopoietic progenitor cells and monocytes (Bautz et al. 2001; Thivierge et al. 2001; Woszczek et al. 2008a). We thus determined whether LTD_4 treatment caused CysLTR1-mediated chemotaxis in human Th2 cells by using a chemotaxis assay. This technique required considerable optimisation; we originally started testing for chemotaxis using the 96-well ChemoTX plate system from NeuroProbe and a Boyden chamber using protocols developed by James Pease (Imperial College).

With this technique, fully differentiated *in vitro*-cultured human Th2 cells were collected and counted. Cells were spun down at 1200 rpm for 5 minutes and were re-suspended in 0.1% BSA (Bovine Serum Albumin) in RPMI medium to obtain 200,000 cells per 20 µl. Wells were pre-incubated with 30 µl 1% BSA in RPMI medium for 30 minutes in order to avoid chemokines and leukotrienes binding to the plasticware of the plate. Following incubation, 30 µl were aspirated and wells were left to dry. Chemokines and leukotrienes were prepared in dilution series from 100 mM stock solution, in 0.1% BSA in RPMI medium and 31 µl were used per well. The membrane layer was then carefully laid down and 20 µl containing 200,000 cells were placed

above each well as a drop on the membrane. The lid of the chamber was then placed on the plate and the chamber was incubated in a damp container for 5 hours at 37°C. Following incubation, drops of cells on top of the membrane were scraped off using a cell scraper and the membrane removed carefully. Each sample in each well was then counted for migrated cell numbers using a haemocytometer. Alternatively, cells were counted using luminescence. Cells were scraped off and the membrane was removed before transferring migrated cells/chemokine mix from each well into a white 96-well plate by centrifuging 5 minutes at 1200 rpm. 30 µl cell glow were added per well before covering the white plate with a plastic film and rotating it for 5 minutes before reading cell counts using a luminescence plate reader. Luminescence of each sample was determined by subtracting luminescence counts from wells containing no cells. Although the assay was successful and migrated cells were visible under the microscope, we could not fully determine whether the cells had dropped due to the membrane pore size being too large or whether they had actually migrated towards leukotrienes. Additionally it was very difficult to quantify the migrated cells as they stuck to the plastic. The luminescence reading technique did not give us accurate enough data and we proceeded to optimize the chemotaxis assay for better results.

While researching a way to optimise our chemotaxis assay and studies which used human T cells in migration assays, we found a range of methods with a lot of variation in techniques and equipment. Variations included assay type and assay system, resuspension buffer for cell preparation, cell numbers in the assay, incubation times and cell counting and analysis methods. A study by Zhou *et al*, looking at the migration of Th17 cells, used a Transwell assay with a membrane of 3 µm pore size and an assay

incubation time of 24 hours (Zhou et al. 2011) . Another by Vijayanand (Vijayanand et al. 2010) prepared CD45RO+CD4+ memory T cells overnight, before using 500,000 cells resuspended in HBSS with BSA and HEPES for four hours in a 96-well chemotaxis chamber with a membrane of 3 µm pore size, before counting migrated cells using a haemocytometer (Vijayanand et al. 2010). Perez-Novo *et al.* used a 96-well ChemoTx plate with a membrane of 5 µm pore size for their Th2 cells resuspended in X-VIVO media. The group incubated the Th2 cells for an hour before centrifuging the plate to collect any cells underside of the filter and analysing the data using flow cytometry (Perez-Novo et al. 2010). Using *in vitro*-cultured T cells isolated from mice, Mira *et al.* used a transwell assay with a membrane of 5 µm pore size, using T cells resuspended in RPMI 1640 with FCS and anti-CD3/CD28 and incubated overnight. Following a 2-hour transwell incubation, cells were analysed by flow cytometry (Mira et al. 2008).

We found that there was very limited data available in the literature regarding the optimisation of chemotaxis assays for human lymphocytes until a study by Islam *et al.* in 2011, looking at mouse CCL8 and its chemotactic effect on CCR8-expressing inflammatory Th2 cells enriched for IL-5 expression. Since the chemotaxis assay technique was not clearly explained in the methods, we requested additional information from the author Sabina Islam. In their study, the group identified mouse CCL8 as a second mammalian agonist for the known chemokine receptor CCR8. The chemokine was found to specifically induce migration of a recently activated, highly differentiated Th2 cell population enriched in IL-5 and IL-25R, TNF and OX40. Interestingly this was only specific to the mouse system, as human CCL8 did not activate CCR8. The group also suggested a role for mouse CCR8 in mediating chronic

cutaneous allergic inflammation. For their migration experiments, the group used Transwell plates with a polycarbonate membrane for filter of 5 μm pore size. Cells were resuspended in RPMI buffer and rested for 4 hours in low rIL-2 concentration prior to running the assay. Cells were then harvested, washed in PBS then used at a concentration of 500,000 cells per 100 μl in a buffer made of RPMI, HEPES and BSA. The amount of chemokines or leukotrienes loaded in the well was 600 μl and the assay was incubated for 2 hours at 37°C and 5% CO_2. The counting method was also different than those seen in other studies. Here, 15 μm beads were added to the migrated cells and acted as a standard for counting cells by flow cytometry (Islam et al. 2011).

Using this method we managed to optimise the chemotaxis assay using Th2 cells and proceeded with testing various leukotrienes and chemokines with varying concentrations.

Firstly we performed the chemotaxis assay using LTD_4 at a wide range of concentrations using the exact method described above. The results for this are shown in **figure 4.1**, in which Th2 cells migrated towards LTD_4 in a concentration-dependent manner. The optimum concentrations observed for Th2 cell migration were between 3 nM and 10 nM LTD_4, which strongly resembles the EC_{50} for LTD_4 in the calcium mobilization studies. Additionally, the migration observed was inhibited when incubating the Th2 cells with specific $CysLT_1$ receptor antagonist MK571 prior to running the assay.

Further experiments comparing LTD_4 with known chemoattractants revealed that the chemokine SDF-1α, which has been shown to induce migration in human lymphocytes, particularly both Th1 and Th2 cells (Siveke and Hamann 1998), caused a robust

chemotactic response in human Th2 cells (**Figure 4.2**). In contrast CCL18, which has been reported to be selectively chemotactic for human Th2 cells, did not cause chemotaxis (**Figure 4.2**). LTD_4 was found to be a potent chemoattracting mediator to fully polarized Th2 cells and induced concentration-dependent chemotaxis of human Th2 cells with a classical bell-shaped curve (**Figure 4.2**).

Additional experiments were performed to compare the migration effect of LTD_4 in comparison to LTE_4. As shown in **figure 4.3**, LTD_4 was more chemotactic to Th2 cells than LTE_4.

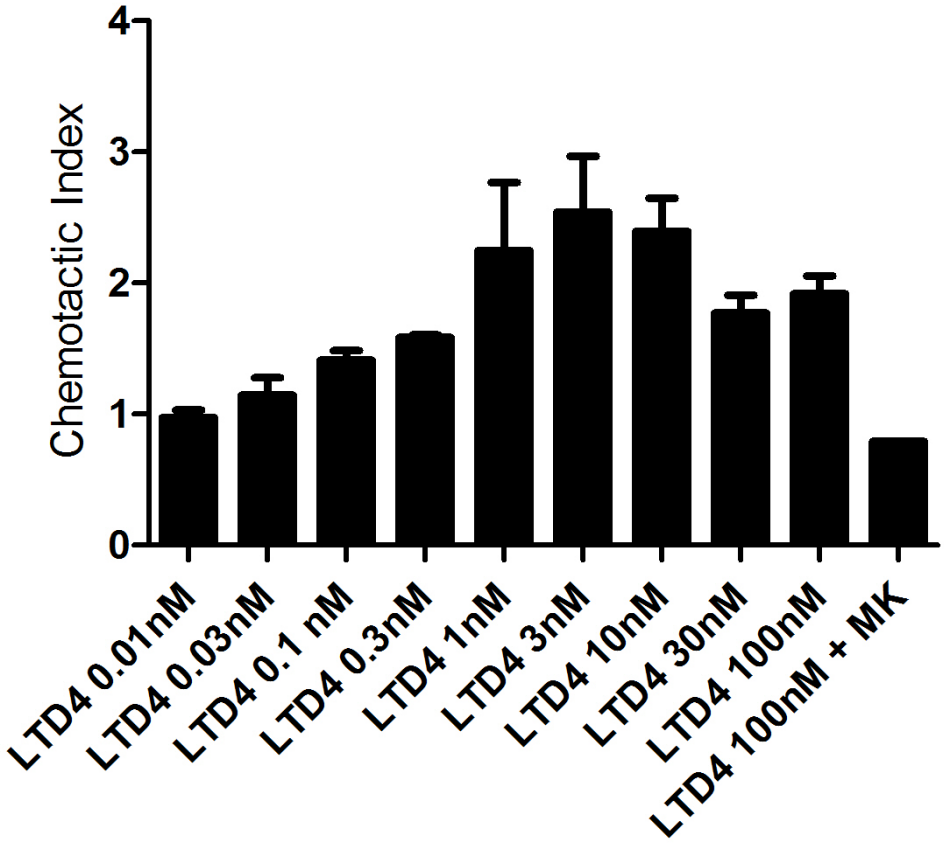


Figure 4.1 LTD_4 is chemotactic for Th2 cells in a concentration-dependent manner. Chemotactic agents were assessed in a Transwell-based system. and cells were allowed to migrate for 2 hours. Different concentrations of LTD_4 were used as shown. CysLT1R antagonist MK571 was used at 100nM concentration and inhibited chemotaxis. Data shown are means ± SEM of 3 independent experiments with different donors.

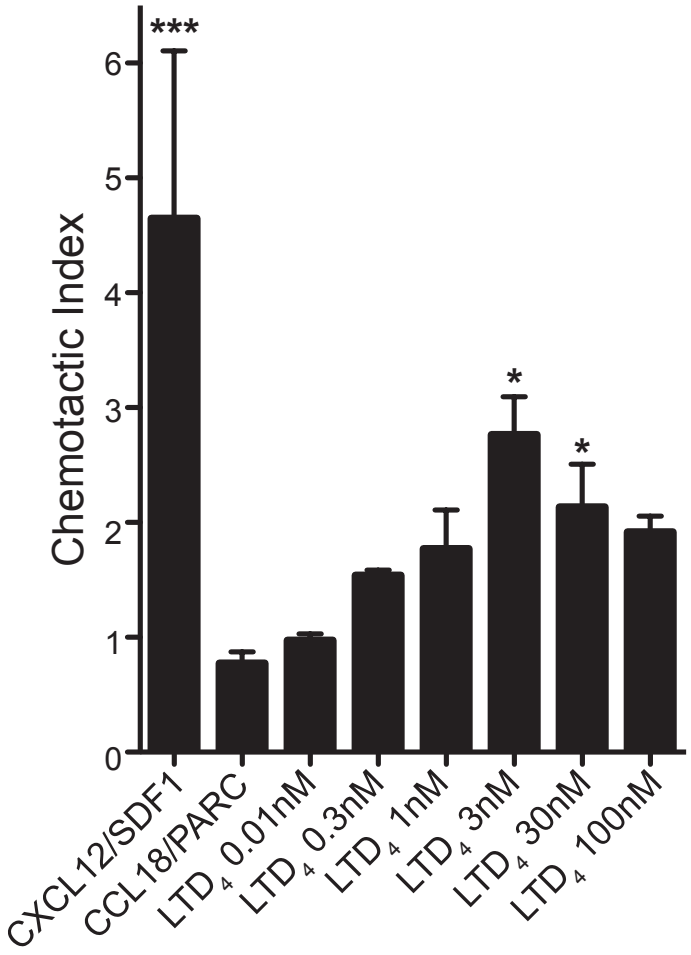


Figure 4.2 Comparison of LTD_4 and controls on the migration of Th2 cells. Chemotactic agents were assessed in a Transwell-based system. Cells were allowed to migrate for 2 hours. Different concentrations of LTD_4 were used as shown. Control chemokines CXCL12 and CCL18 were used at 10nM concentration. Data shown are means ± SEMs of 3 independent experiments with different donors. ***P < 0.001 and *P < 0.05, 2-way ANOVA with Bonferroni post test.

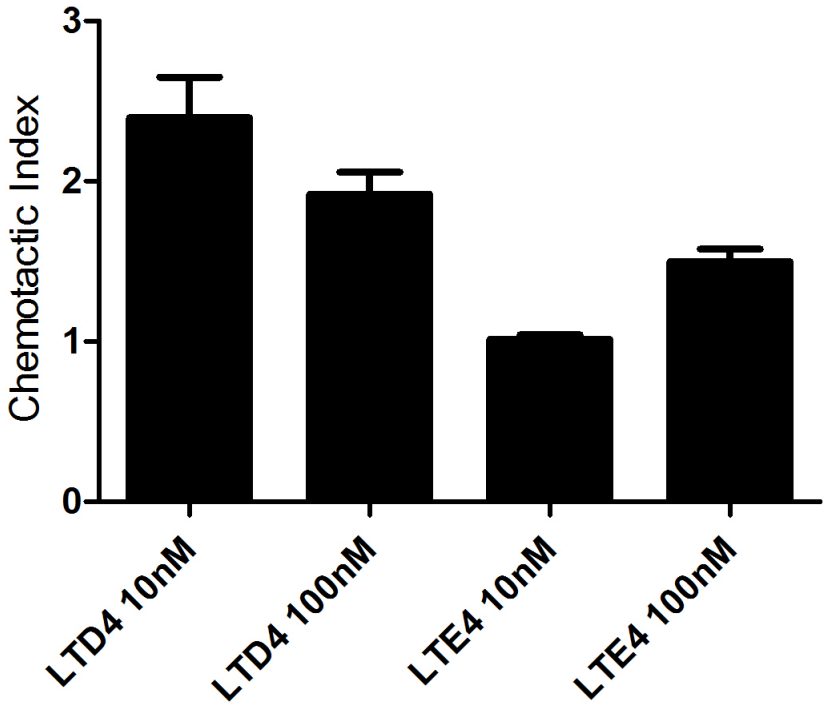


Figure 4.3 LTD_4 is more chemotactic for Th2 cells than LTE_4. Chemotactic agents were assessed in a Transwell-based system. Cells were allowed to migrate for 2 hours. Different concentrations of cysteinyl leukotrienes were used as shown. Data shown are means ± SEMs of 3 independent experiments with different donors.

Based on evidence that PGD2 binds to Th2-specific CRTh2 receptor, we decided to test PGD2 in our chemotaxis assay and compared its effect to that of LTD_4 at various doses.

PGD2 has previously been reported to bind to receptor CRTH2, a Th2-specific marker also expressed in basophils and eosinophils (Nagata et al. 1999a; Nagata et al. 1999b). In a study by Hirai *et al.*, they showed that PGD2 induced chemotaxis. Chemotaxis was shown to be specific to Th2 cells and not Th1 cells and was shown to occur via the CRTH2 receptor and not the known PGD2 receptor DP (Hirai et al. 2001).

In their experiments, human Th1 and Th2 lines were generated from PBMCs from healthy adults and stimulated with a purified protein derivative of Mycobacterium tuberculosis in the presence of IL-12 and IFN-y for Th1 cells, and with an extract of Dermatophagoides in the presence of IL-4, anti-IFN-y and anti-IL-12 antibodies for Th2 cells. Both cell lines were expanded by IL-2 and used after 7-14 days. Some of the chemotaxis experiments used Th2 cells enriched by sorting with an anti-CRTH2 mAb (BM16) prior to the assay. The assay was performed on a 96-well microchemotaxis chamber, using a 5 μm pore filter. Cells were prepared in RPMI 1640 with FCS and HEPES and incubated with chemoattractants for an hour, before being counted by flow cytometry (Hirai et al. 2001).

Similarly, Th2 cells have been reported to respond to PGD2 produced in mast cells supernatants (Gyles et al. 2006). Gyles *et al.* investigated the role of PGD2 and its receptor CRTH2 in the chemotactic response of Th2 cells to supernatants collected from immunologically activated human mast cells. In this *in vitro* setting, the group found Th2 cells to chemotax to PGD2. Treatment of mast cells with cyclooxygenase inhibitor diclofenac substantially inhibited the production of PGD2 by mast cells, which

therefore inhibited the production of CRTH2+ CD4+ Th2 cell stimulatory activity. Addition of PGD2 to the conditioned media restored the ability of the mast cell supernatants to induce chemotaxis in Th2 cells. Experiments using CRTH2 antagonist ramatroban in the supernatants inhibited the chemotactic response. The use of selective thromboxane-like prostanoid (TP) antagonist SQ29548 showed no significant inhibitory effect on the migration of CRTH2+ CD4+ Th2 cells, which confirmed CRTH2 to be the receptor mediating the chemotactic response (Gyles et al. 2006).

In our study, we used three different doses of PGD2, 0.1 nM, 1 nM and 10 nM, and tested these in a similar way to the various doses of LTD_4. **Figure 4.4** shows that, in our system, we were unable to reproduce the results reported by Gyles *et al.* and *Hirai et al.* In our experiment, PGD2 had very little effect on Th2 cells. This lack of chemotactic response was observed with all three tested concentrations. This could be due to differences in the *in vitro* human Th2 cell differentiation system or variations in the chemotaxis assay.

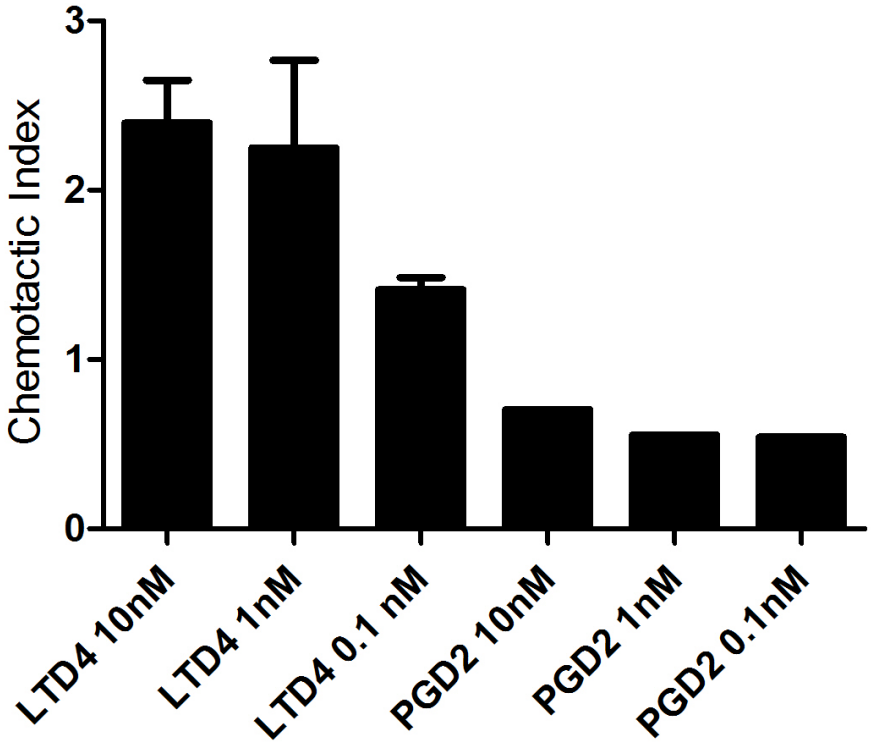


Figure 4.4 Th2 cells do not migrate to PGD2. Chemotactic agents were assessed in a Transwell-based system. Cells were allowed to migrate for 2 hours. Different concentrations of LTD_4 and PGD2 were used as shown. Data shown are means ± SEMs of 3 independent experiments with different donors.

Following functional studies and migration assays, we investigated the role of LTD_4 on the proliferation of Th2 cells. Other groups such as Yuan *et al*. have also investigated the role of LTD_4 on proliferation. Indeed, Yuan *et al*. showed that LTD_4 had no effect on the proliferation of endothelial cells, in contrast to vascular endothelial growth factor VEGF, which significantly increased the number of proliferated cells. The methods used (Trypan blue exclusion assay and BrdU incorporation assay) were different than the technique we used. In this study, the group showed that, although no effect was found on proliferation, LTD_4 was found to induce migration of endothelial cells in wound healing, as effectively as VEGF (Yuan et al. 2009).

Using a thymidine incorporation assay, we sought to determine whether LTD_4 had any effect on T cell proliferation. The assay functions by utilizing a strategy wherein a radioactive nucleoside, 3H-thymidine, is incorporated into new strands of chromosomal DNA during mitotic cell division. A Microplate Scintillation and Luminescence Counter (top count NXT) was used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division that has occurred in response to a test agent, in our case LTD_4. This assay allowed for direct measurement of proliferation.

Both fully differentiated Th1 and Th2 cells were tested and thymidine was added 18 hours prior to running the assay on both CD3/CD28-coated and uncoated wells. Unfortunately, we could no detect any change between Th1 and Th2 control cells and cells with LTD_4. Additionally, Th2 cells appeared to still be activated at the time of the assay, which made it difficult to obtain accurate results. Unfortunately we observed similar results when we repeated the assay (data inaccurate and not shown).

We also tested the effect of LTD_4 on Th1 and Th2 *in vitro* differentiation cultures to examine whether LTD_4 had any direct effect on the Th1 and Th2 differentiation and cytokines expression. Following 21 days of culture with and without LTD_4, Th1 and Th2 cells with and without LTD_4 were stained intracellularly for various Th1- and Th2-specific cytokines. No significant effect was observed on cytokine productions in either Th1 or Th2 cells that had been cultured with LTD_4, as seen in **figure 4.5**.

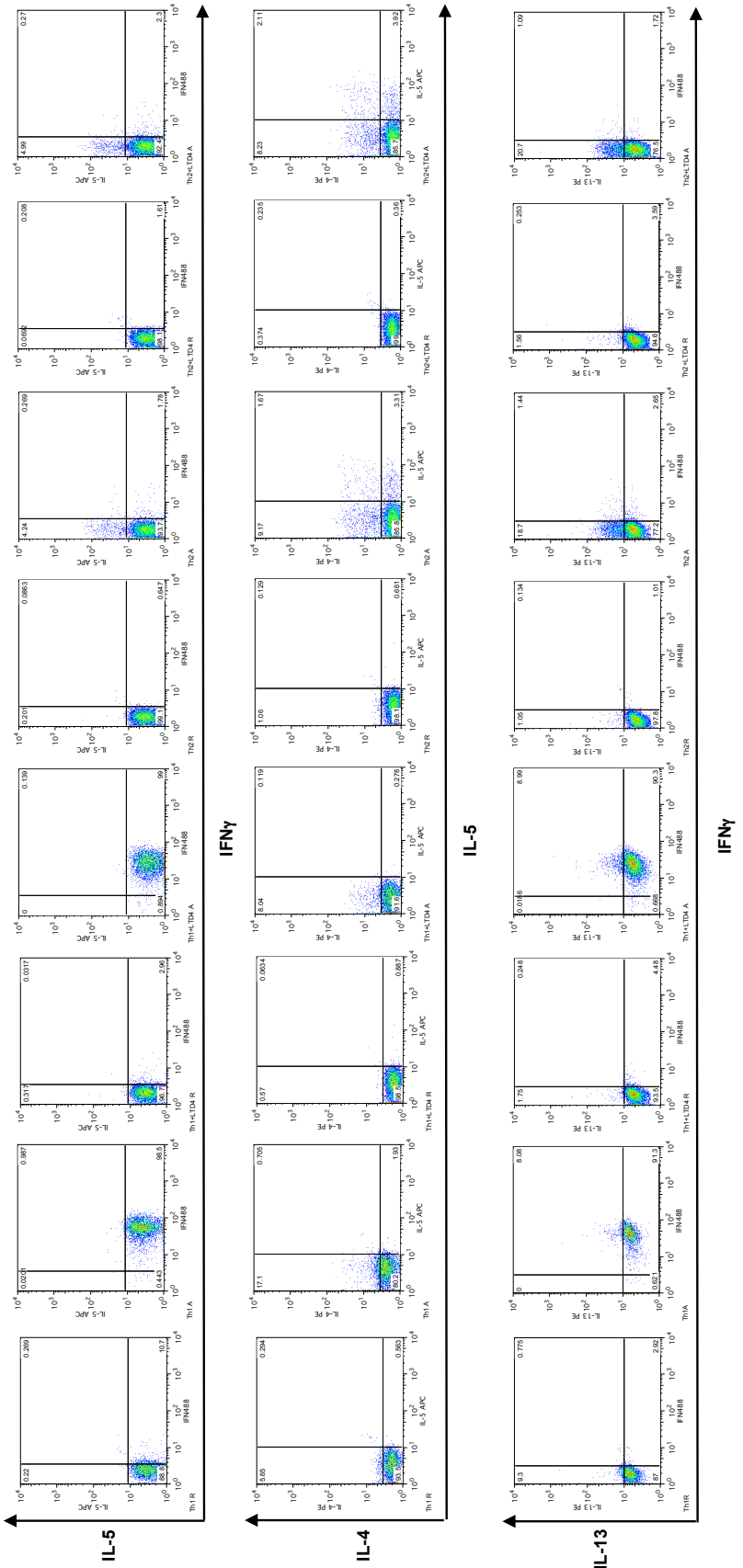


Figure 4.5 LTD_4 has no effect on Th1- or Th2-specific cytokines during differentiation. Th1 and Th2 cells were cultured and differentiated with or without 100nM LTD4 and tested for intracellular cytokine staining at Day 21. No significant effect was observed. Data shown are representative of 3 independent experiments with different donors.

Following the results obtained by flow cytometry using Th1 and Th2 cells cultured with LTD_4 and the lack of detection of any change in cytokine expression, we used RT-PCR to check for any cytokine expression changes at mRNA level. Using fully differentiated Th1 and Th2 cells at Day 21, we set up a time-course at time 30 min, 1 hour, 4 hours and 24 hours, with and without LTD_4. Samples were harvested at each time point and mRNA was isolated. With these samples, we looked at mRNA expression for Th1- and Th2-specific cytokines, such as IFN-γ, IL-4, IL-5 and IL-13 to identify potential immediate changes in cytokine production. We found that the addition of LTD_4 in our Th1 and Th2 cultures had no immediate or later effect on the mRNA expression of Th1- and Th2-specific cytokines, as seen in **figure 4.6**. IFN-γ was found mainly on Th1 cells and the expression was higher at 30 min and 1 hour, and then decreased at 4 and 24 hours. LTD_4 had no effect on the expression. IL-4, IL-5 and IL-13 each showed a similar pattern of expression, with a high expression at 30 min and a progressive decrease in expression at 1, 4 and 24 hours, with little or no expression left at 24 hours.

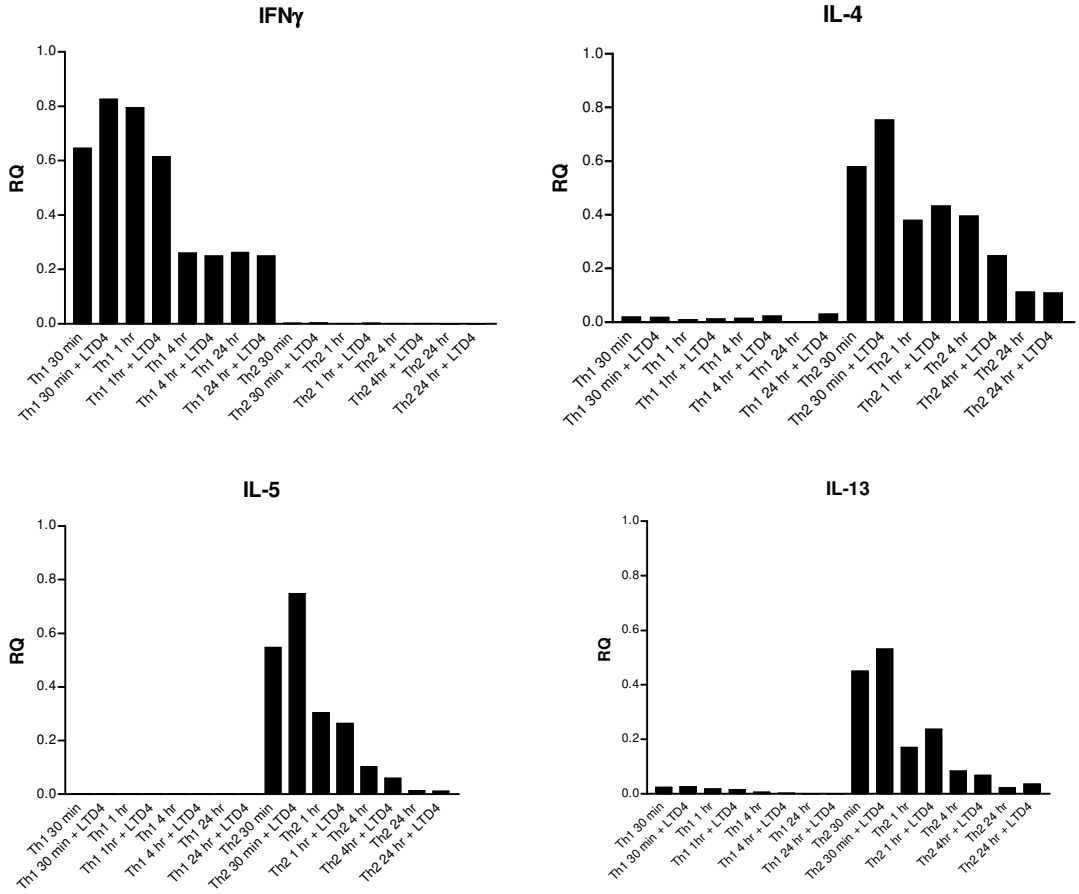


Figure 4.6 mRNA expression following Th2 cell culture with LTD_4. No LTD_4 or LTD_4 was added to Th1 and Th2 cells, which were harvested after an incubation of 30 min, 1 hour, 4 hours and 24 hours to identify whether LTD_4 had any effect on cytokine mRNA expression. Data are representative of three experiments, using at least three technical replicates.

4.3 Summary

In previous experiments, we have shown that the human $CysLT_1$ receptor is coupled to both Gαi/o and Gαq/11 in our in vitro-differentiated human Th2 cells system. This corresponds to previous findings that the receptor is partially coupled in native systems but only coupled to Gαq/11 in recombinant systems.

In these experiments we have shown that Th2 cells migrate to LTD_4 in a dose-dependent manner and this is the first report that human Th2 cells can migrate to cysteinyl leukotrienes. We also showed that antagonist MK571 inhibited the chemotactic response. We have also shown that the addition of LTD_4 to Th1 and Th2 cells during proliferation and differentiation did not have any significant effect on cytokine expression (at least for those cytokines that we examined). We also found that LTD_4 had no significant effect on proliferation using proliferation assays, however this assay was not fully accurate.

CHAPTER 5
RESULTS: EFFECT OF LTD4 ON GENE EXPRESSION IN HUMAN TH2 CELLS

Chapter 5 Results – Effect of LTD_4 on gene expression in human Th2 cells

5.1 Introduction

5.1.1 Comprehensive analysis of gene expression changes in response to CysLTs

The studies performed in the previous chapter showed that CysLTs acting via $CysLT_1$ receptor function to cause migration of human Th2 cells in chemotaxis assays. However we failed to detect any noticeable effects on Th2 cell proliferation, differentiation or cytokine secretion (at least for the Th1/Th2 cytokines examined). To comprehensively examine the effect of CysLT treatment on transcription in Th2 cells we decided to investigate the effect of LTD_4 treatment on gene expression levels using microarrays. The rationale for the use of microarrays was that it would allow us to examine the vast majority of genes in the genome in an unbiased manner. Previous studies have investigated the function of CysLTs on other cell types using a transcriptomic approach (Uzonyi et al. 2006; Woszczek et al. 2008b).

Woszczek *et al.* have undertaken a similar study in human monocytes, in which they sought to determine the gene expression signature response of human monocytes exposed to CysLTs, in order to elucidate the signalling pathways involved in monocyte activation. Using human elutriated monocytes that had been stimulated with 100 nM LTD_4 for 30 minutes, they performed a microarray using the Affymetrix HG U133 plus 2.0 arrays. The group found that, at 30 minutes, LTD_4 induced seven genes belonging to families of transcription factors and signalling molecules. Of the seven genes, five were further analysed by RT-PCR. The expression of mRNA for transcriptional regulators, such as FOSB, EGR2, EGR3, NR4A2, and TSC22D3, was quickly induced. The two most highly upregulated genes induced by LTD_4 and confirmed by RT-PCR were FBJ

murine osteosarcoma viral oncogene homolog B (FOSB) and Egr2, with the highest increase observed between 30 and 60 minutes and returning to baseline after 2 hours. These data therefore showed that LTD_4 activates the expression of immediate-early genes in human monocytes (Woszczek et al. 2008b).

To our knowledge, the only other comparable microarray data come from a study by Uzonyi *et al.*, in which they determined the gene signature of human umbilical vein endothelial cells (HUVEC) in response to LTD_4. Using thrombin signalling through PAR1 as a positive control, they found that CysLT2R activation induces early gene signatures that resemble those after PAR1 activation and that the combined actions of LTD_4 and thrombin further stimulate gene expression. The group identified 37 genes that were significantly induced after 60 minutes of stimulation with LTD_4, and most of these were also found to be upregulated by thrombin. Interestingly, the same families of transcription factors (EGR, activating transcription factor) were upregulated, such as COX2, EGR1, EGR2 and NR4A2. Some of the genes found in HUVECs also compared to the ones identified in human monocytes. However, LTD_4-induced genes in HUVECs were the result of $CysLT_2$ receptor activation and signalling, as these cells only express CysLTR2. Induction of several of the genes was found to be resistant to specific CysLTR1 antagonists (Uzonyi et al. 2006).

We therefore investigated the effect of LTD_4 on various time-points by microarrays (30 min, 4 hours, 24 hours) and found several upregulated genes, which were similar to those found by Woszczek and Uzonyi.

5.2 Results

5.2.1 Experimental design and exploratory analyses

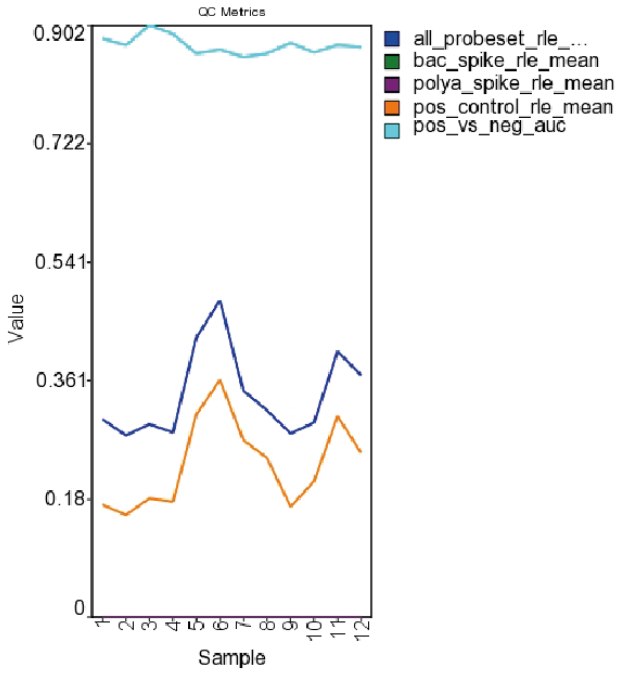
To examine the effect of CysLTs on gene expression we aimed to analyse Th2 cells treated with and without 100 nM LTD_4 at various time points. LTD_4 was chosen as the agonist since it was the most potent in the other assays examined such as calcium, cAMP and chemotaxis. For this study, we decided to focus on fully differentiated Th2 cells 21 days after naïve cell isolation. Prior to the experiments, Th2 cells were incubated with L-cysteine, a known inhibitor of the LTD_4 to LTE_4 conversion dipeptidase, in order to sustain the effect of LTD_4 during treatment. The cells were then incubated with or without 100 nM LTD_4 and harvested at various time points – 30 min, 1 hour, 4 hours and 24 hours - for RNA extraction. This allowed us to explore the effect that LTD_4 had on Th2 cells when present for different time periods, from early effects to late effects. Two biological replicates were performed on different donors. RNA extraction was performed using the Qiagen miRNeasy protocol and the RNA samples were processed for microarray experiments as described in the Materials and Methods section.

The microarray platform used in these experiments was the Affymetrix Human Gene 1.0 ST GeneChip, which examines 36,079 RefSeq transcripts including 21,014 well-annotated genes. The RNA isolated was of high quality as measured by bioanalyser RNA integrity number (RIN) and the labelled cRNA samples met all of the recommended Affymetrix quality control criteria prior to loading onto the GeneChips (data not shown). After array processing and scanning, the data was imported into Partek Genomics Suite as CEL files (Partek Inc.) for further data analysis. Partek is a

statistical software package that performs multi-way ANOVA analyses of microarray data. The data was pre-processed using Robust Multi-array Average (RMA) and quantile normalisation using the standard Partek workflow. Quality control analyses are shown in **figure 5.1**. Panel **A** shows that the intensity signals for the positive and negative controls were similar across all arrays with no substantial outliers. Panel **B** shows that the signal intensity distribution of all probes was similar in each microarray. Collectively these quality control data suggest that all of the samples were of good quality and that the microarrays have all functioned correctly. Since the microarray data passed the quality control steps, the normalised data containing multiple probe sets for each transcript was summarised to a gene level analysis using the standard Partek workflow. After the gene-level summarisation, principal component analysis (PCA) was performed in Partek as an exploratory step in the data analysis (**Figure 5.2**). PCA allows visualisation of high-dimensional data to examine the major sources of variation in the data. The first three principal sources of variation (components) are represented by the axes, and each spot on the graph represents a single microarray sample (**Figure 5.2**). It is clear from the PCA analysis that the major source of variation relates closely to the time-point of sampling (PC1: 36.4%). The next most obvious source of variation is a donor effect (PC2: 13.8%). There is no obvious effect attributable to LTD_4 treatment at this level of analysis. The nature of the experimental design means that we cannot discriminate between a true donor effect or an artifactual batch effect since the biological replicate experiments and RNA isolations were performed on different dates. Using the multi-way ANOVA model in Partek it is possible to remove the effect of batch/donor to allow for better interpretation of the

dataset. Batch effect removal was performed in Partek and exploratory PCA analysis performed on the transformed data (**Figure 5.3**).

A



B

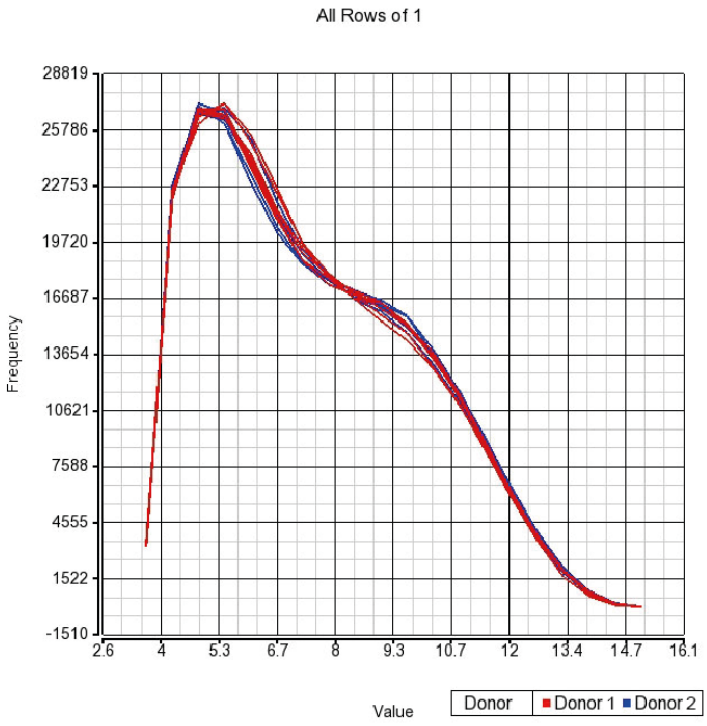


Figure 5.1 Quality control of microarray data. (A) Intensity of positive and negative control signals across all arrays. (B) Signal intensity distribution of each array.

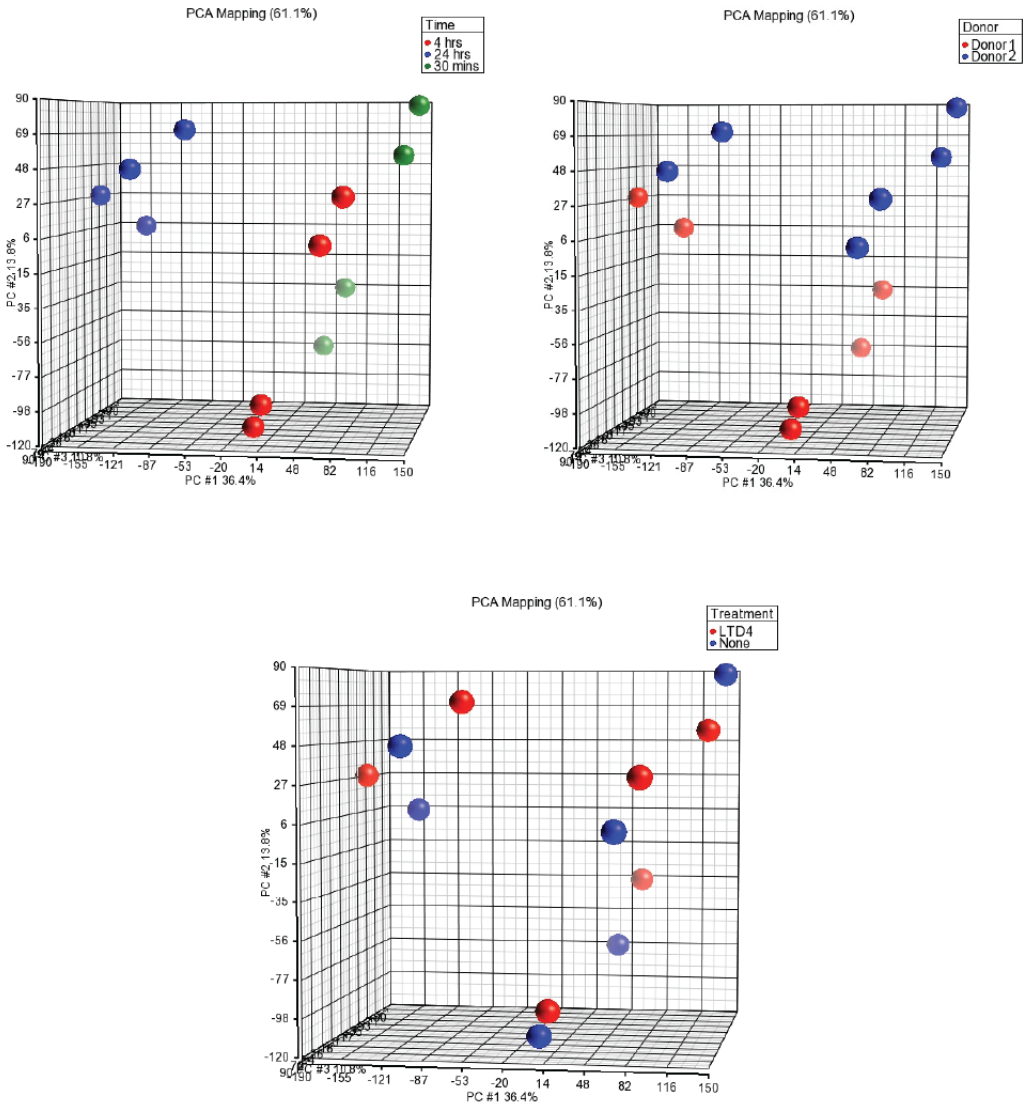


Figure 5.2 Principal component analysis of gene summarised data. The three panels show the same PCA analysis coloured using different criteria as indicated in each legend.

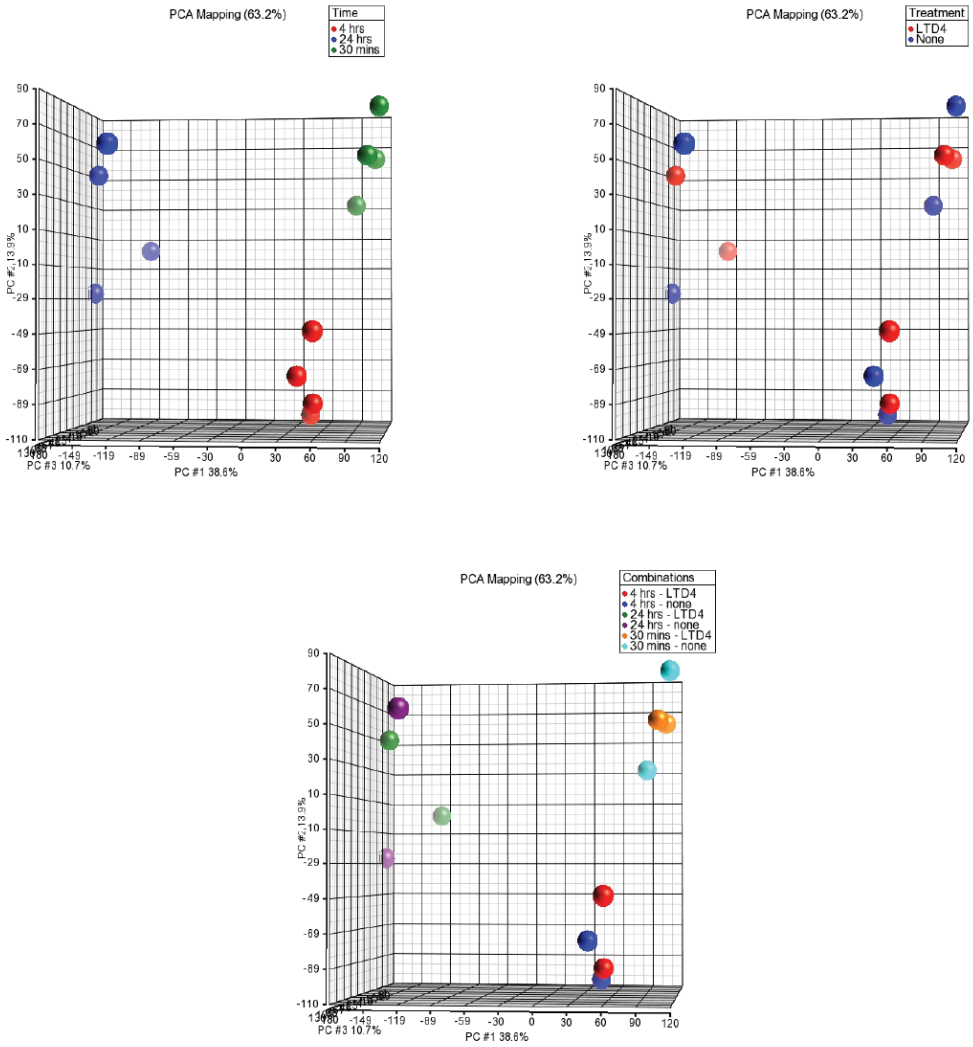


Figure 5.3 Principal component analysis of gene summarised data after batch effect removal. The three panels show the same PCA analysis coloured using different criteria as indicated in each legend.

Figure 5.3 clearly shows that batch effect removal causes a change in the sources of variation detected by the PCA. Importantly it causes the samples discriminated by time to cluster together more than in **Figure 5.2**. The major source of variation is still closely related to the time point of the sample (PC1: 38.6%). However there is still no obvious effect attributable to LTD_4 treatment. Even at this early stage of exploratory analysis it suggests that treatment with LTD_4 does not have a profound effect on the gene expression profile of Th2 cells.

5.2.2 Detection of differentially expressed genes upon LTD_4 treatment

Although the preliminary analyses suggest that LTD_4 treatment does not have a profound effect of global gene expression patterns in human Th2 cells, it does not rule out the possibility that a subset of genes are regulated by LTD_4 signalling. To identify genes that are differentially expressed due to LTD_4 treatment we performed a three-way ANOVA in Partek using time, LTD_4 treatment and donor/batch as factors (see Methods for further details). Donor/batch was considered a random effect and we also included in the ANOVA model an interaction between time and LTD_4 treatment (i.e. LTD_4 treatment may cause different changes in gene expression depending on the time-point investigated). **Figure 5.4** shows the sources of variation detected in the ANOVA analysis. The figure clearly shows that the major source of variation in the dataset is the time of sampling as expected from the PCA analysis. The second most important source of variation is donor/batch. The role of LTD_4 treatment and the interaction between time and treatment both provide very small components of the overall variation seen in the data.

Analysis of the ANOVA results shows that 9057 genes change due to the time-point of sampling ($p<0.05$) including false discovery rate (FDR)). Hierarchical clustering analysis of this subset of genes identifies blocks of genes with similar expression patterns at the various time-points (**Figure 5.5**).

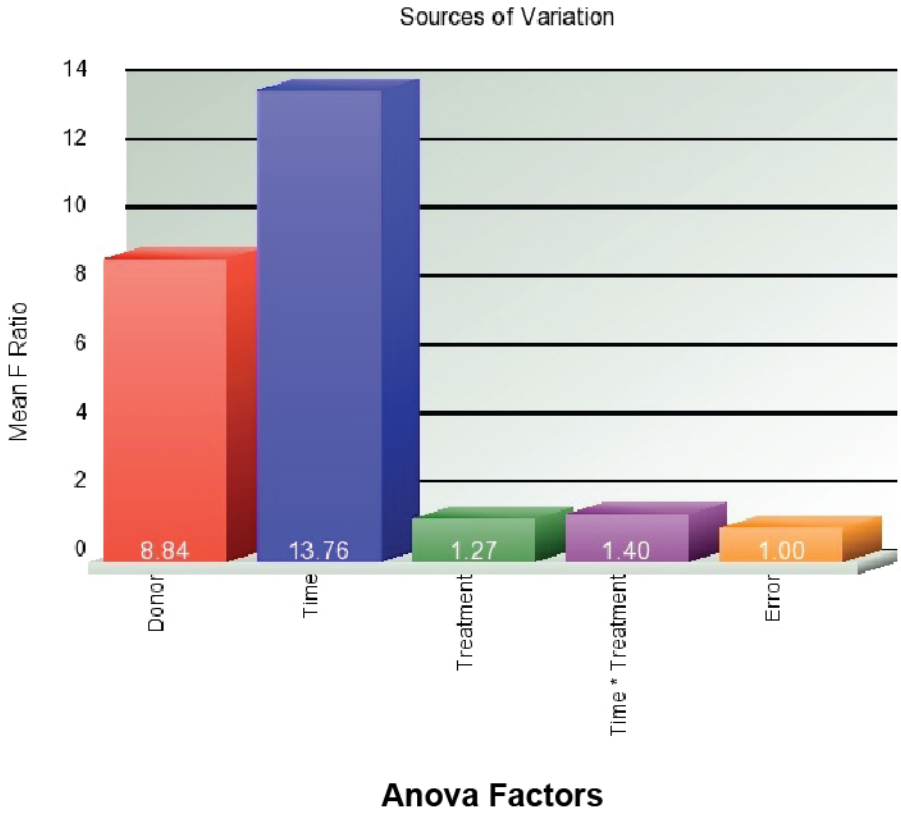


Figure 5.4 ANOVA sources of variation. The relative role of each factor in the variation in the data is shown.

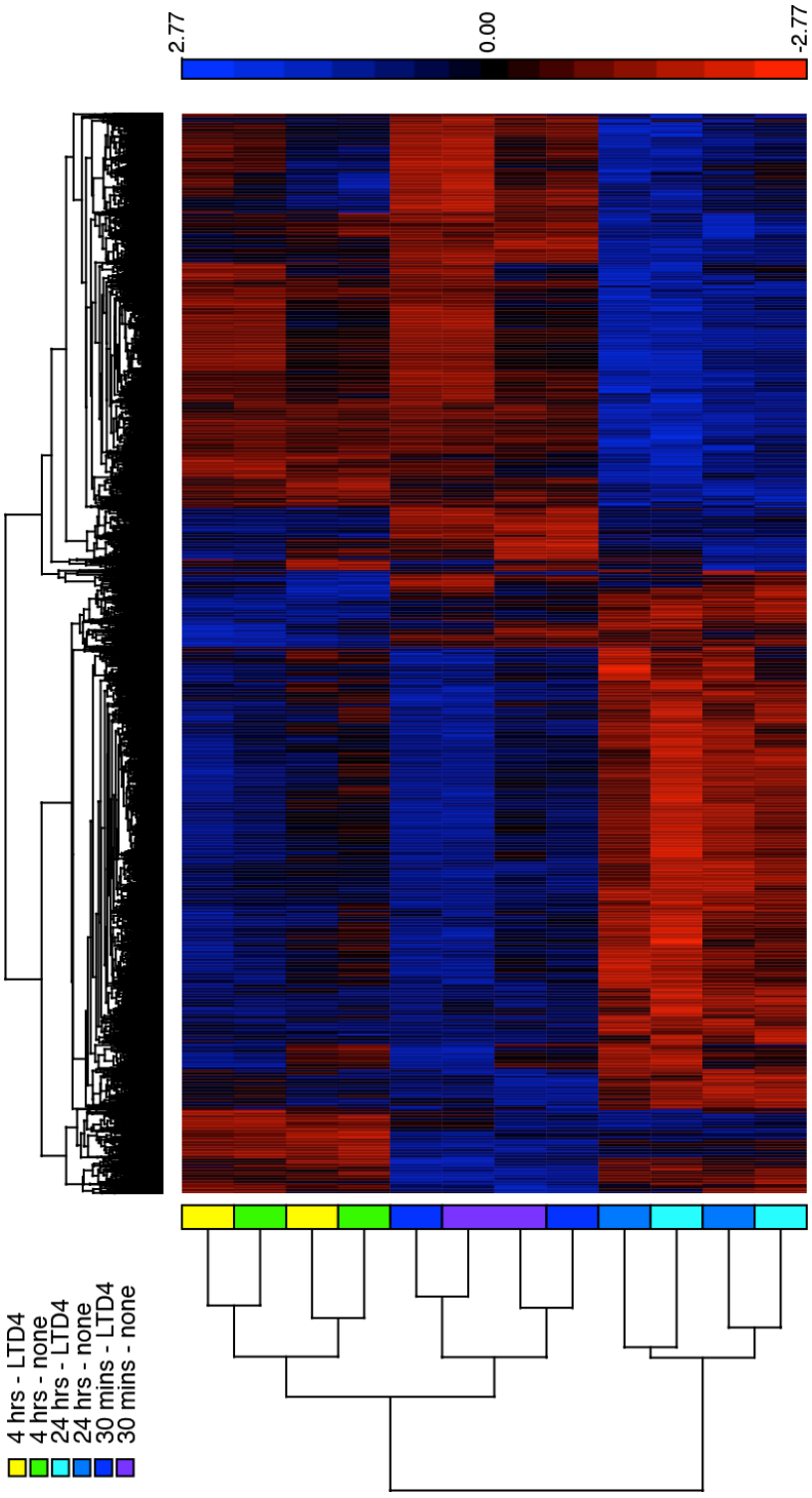


Figure 5.5 Hierarchical clustering of genes differentially expressed due to time.

Whilst the effect of time in culture is interesting in the context of T cell biology it is not the focus of this thesis and therefore we did not investigate these data further. However it is important to point out that these results have implications for the experimental design of future experiments examining global analysis of gene expression in T cells in culture. These cells were essentially 'resting' and yet many genes were altered during the time-course of 24 hours. This means that for future studies examining the effect of a stimulus over time, paired samples (untreated and treated with the stimulus), must be used in order to discriminate between changes due to stimulus and changes due to time.

Examination of the data relating to LTD_4 treatment showed that while 7 genes changed more than two-fold due to treatment, none of these genes reached statistical significance when including FDR (data not shown). It is possible that if we had performed more biological replicates, some of these changes may have reached statistical significance, but due to time and cost limitations such experiments were not performed. However, examination of genes that changed due to the interaction between LTD_4 treatment and time revealed 20 genes that were different between untreated and LTD_4-treated samples at 30 min **(Table 5.1**). Hierarchical clustering of these genes showed a clear pattern of differential expression at the 30 min time-point with the expression of these genes returning to similar levels in both LTD_4-treated and untreated samples at later time-points (**Figure 5.6**, 2 fold; $p<0.05$ including FDR). Examination of changes in gene expression due to LTD_4 treatment at later time-points did not identify any statistically significant differences (data not shown). The gene list identified as differentially expressed after 30 min of LTD_4 treatment contains several

well-known immediate early genes (FOS, FOSB, EGR family) as well as T cell activation marker CD69 and the cytokine TNF-α.

Affymetrix Transcript ID	Gene Symbol	RefSeq	Mean (Untreated 30 min)	Mean (LTD4 30 min)	p-value	Fold-Change
8034694	MIR24-2	NR_029497	5.17856	6.52956	3.76E-05	2.55088
7975779	FOS	NM_005252	6.55434	9.37456	0.0028718	7.06269
7961075	CD69	NM_001781	10.0861	11.7475	0.0043981	3.16337
7933872	EGR2	NM_000399	7.86675	10.1069	0.00766	4.72451
7892567		---	7.26128	6.17626	0.0087855	-2.1214
8055952	NR4A2	NM_006186	6.52855	7.88342	0.0094496	2.55774
8029693	FOSB	NM_006732	6.45904	8.14831	0.0128386	3.22494
8034698	MIR23A	NR_029495	7.16025	8.33351	0.0156821	2.2552
8108370	EGR1	NM_001964	7.72308	10.2386	0.0209553	5.71808
7896526		---	5.10842	6.14126	0.0223156	2.04604
8115831	DUSP1	NM_004417	7.20377	8.74646	0.0258755	2.91336
8118142	TNF	NM_000594	7.73964	8.92597	0.0282552	2.27574
8177983	TNF	NM_000594	7.73964	8.92597	0.0282552	2.27574
8179263	TNF	NM_000594	7.73964	8.92597	0.0282552	2.27574
7896505		---	4.70279	5.73665	0.0293128	2.0475
7922976	PTGS2	NM_000963	7.95715	9.51441	0.0371869	2.94294
8149720	EGR3	NM_004430	5.80703	7.25699	0.0415114	2.732
7896181		---	6.65715	7.66732	0.0435528	2.01414
7895146		---	5.79725	4.74951	0.0437285	-2.06728
8034696	MIR27A	NR_029501	5.59401	6.70597	0.0481581	2.16139

Table 5.1. Genes that are statistically significantly differentially expressed in Th2 cells after 30 mins of LTD_4 treatment. Samples were processed on Affymetrix Gene 1.0 ST arrays and data was normalised and ANOVA performed in Partek as described in Methods. Mean columns show the $\log_2$ of mean array signal for each gene.

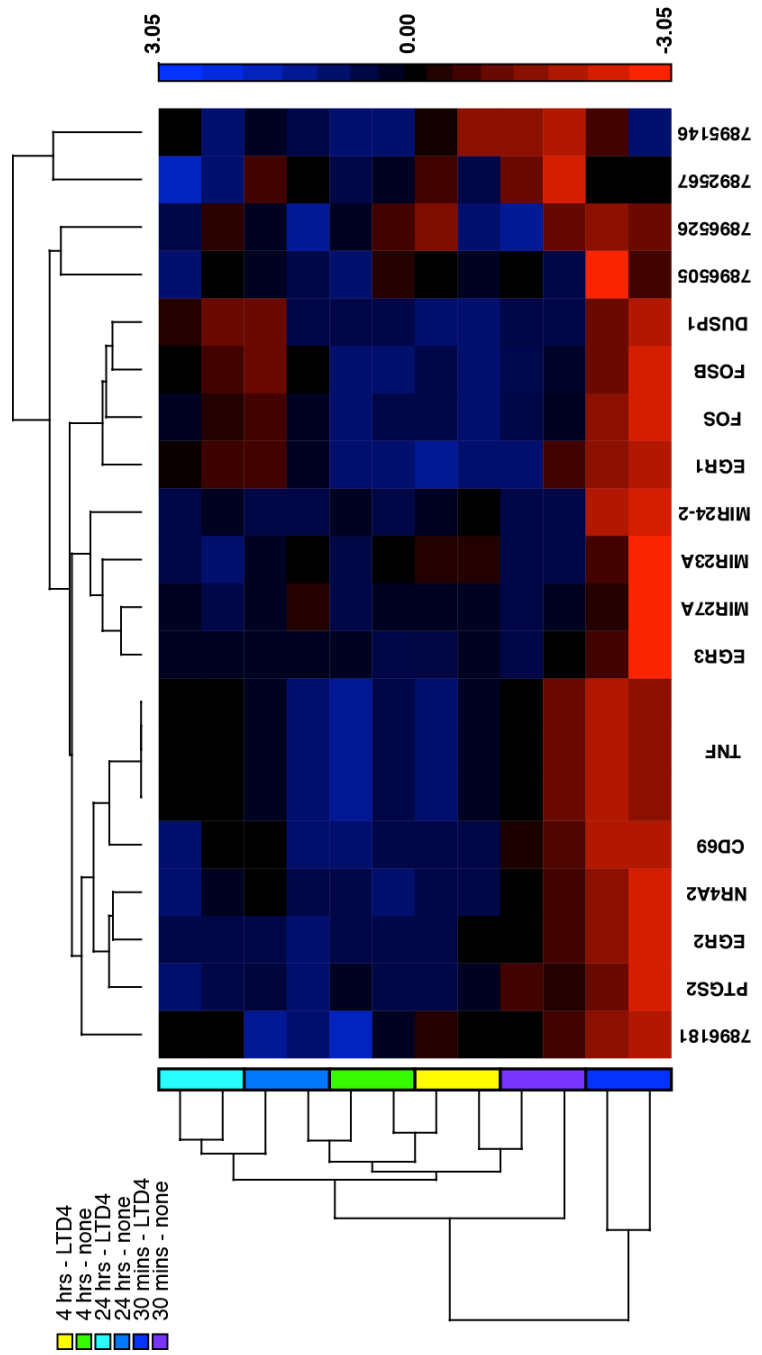


Figure 5.6 Hierarchical clustering of genes differentially expressed due to LTD_4 treatment at 30mins.

While hierarchical clustering and heatmap analysis are useful visualisation tools for microarray studies, other visualisation methods can provide different information in respect to the same data. **Figure 5.7** shows a volcano plot of the data from the 30 min time-point. The X-axis shows the fold-change between untreated and LTD_4-treated samples, the Y-axis shows the $-\log_{10}$p-value from the ANOVA, and each gene is represented by a dot. It can be clearly seen from this analysis that the genes listed in **table 5.1** show both a significant p-value and a high fold change compared to the vast majority of genes that do not change in response to treatment with LTD_4. Interestingly it is also clear from this representation that all of the statistically significantly different genes are upregulated in response to LTD_4 with very little evidence for gene downregulation.

While the Volcano plot is a useful visualisation tool it does not provide information regarding the level of expression of genes of interest (i.e. a gene could have a high fold change and significant p-value and still be expressed at very low levels). **Figure 5.8** shows a scatter plot of the data from the 30 min time-point where the X-axis is the mean gene expression level for each gene in LTD_4 treated samples and the Y-axis is the mean gene expression level in untreated samples. Once again it is clear from this representation that the genes identified as different due to LTD_4 treatment are upregulated rather than downregulated. Interestingly however the scatter plot also provides an estimate of the actual level of gene expression. Genes such as CD69 and MIR21 are expressed at very high levels in comparison to most genes, whereas MIR24-2 is expressed at very low levels and therefore less likely to be a biologically meaningful change in expression.

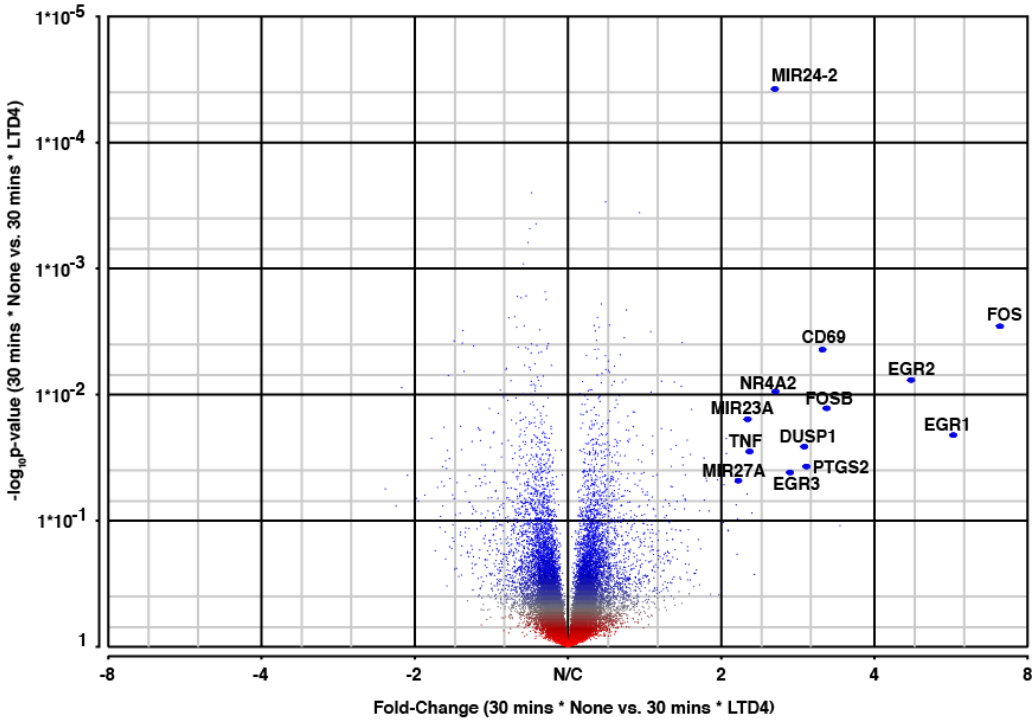


Figure 5.7 Volcano plot of gene expression at 30 min. X-axis shows fold change in expression between untreated and LTD4-treated samples. Y-axis shows -$\log_{10}$(p-value).

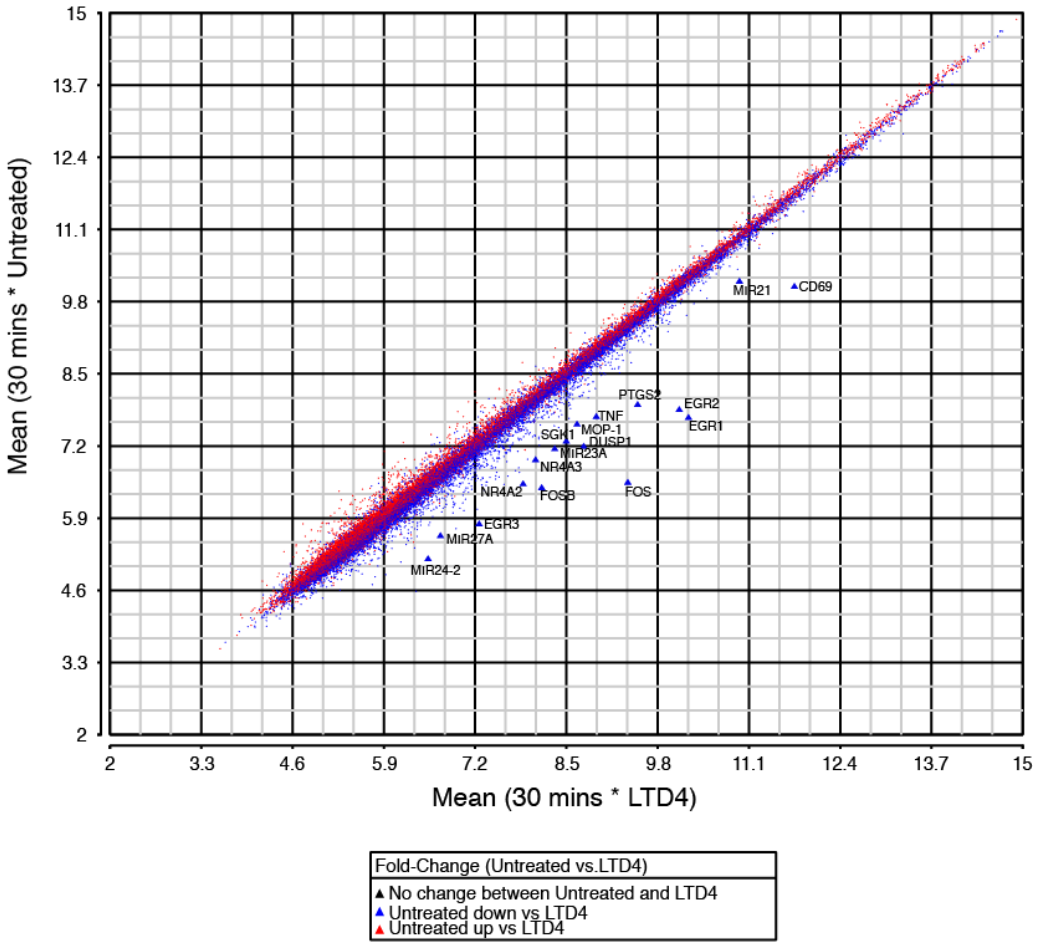


Figure 5.8 Scatter plot analysis of gene expression at 30 min time-point. Each gene is represented by a dot. Genes are colours by fold-change between Untreated and LTD_4-treated samples. Data is the log_2 of mean signal intensity for each gene. Blue genes are higher in LTD_4-treated samples, red are higher in untreated samples.

Collectively these data suggest that LTD_4 functions to upregulate a small group of immediate early genes, some microRNAs and markers of activation.

5.2.3 LTD_4 regulated genes

The genes identified in the last section fall into several functional categories and have different expression kinetics in a variety of other cell types. **Figures 5.9** and **5.10** show the individual expression profile of each gene identified as being regulated by LTD_4.

Genes identified in our system are FOS, EGR1, EGR2, EGR3, FOSB, CD69, NR4A2, DUSP1, TNF, PTGS2, Mir23A, Mir24-2, Mir27A. Of these, we found a 4- to 6-fold change for FOS, EGR1 and EGR2; and a 2- to 4- fold change for the others. All of these genes were found to be upregulated following 30 min treatment with LTD_4. Here we describe the effect of LTD_4 on the genes.

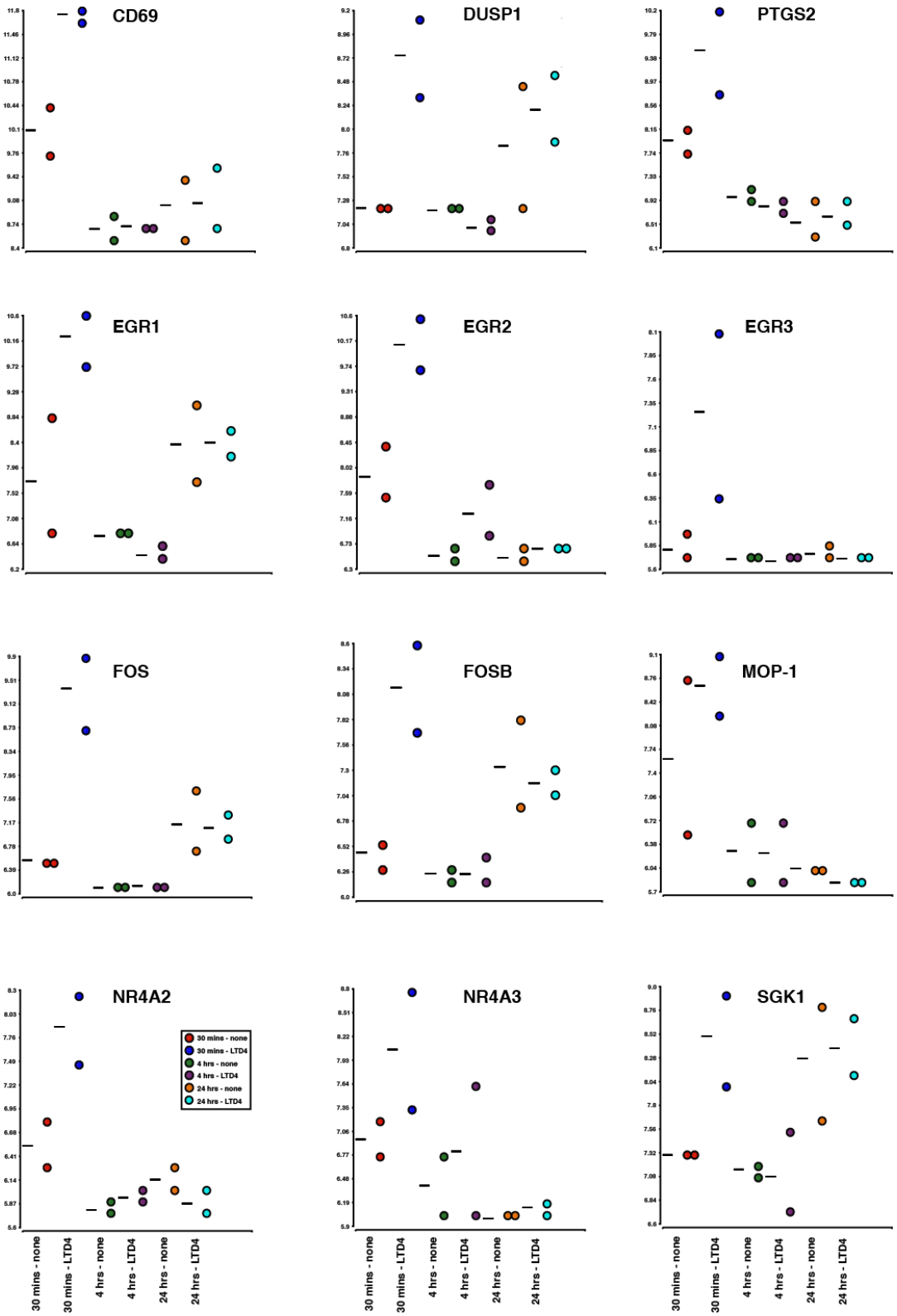


Figure 5.9 Expression profile of genes regulated by LTD_4. X-axis shows treatment and time combinations. Y-axis shows $\log_2$ signal intensity.

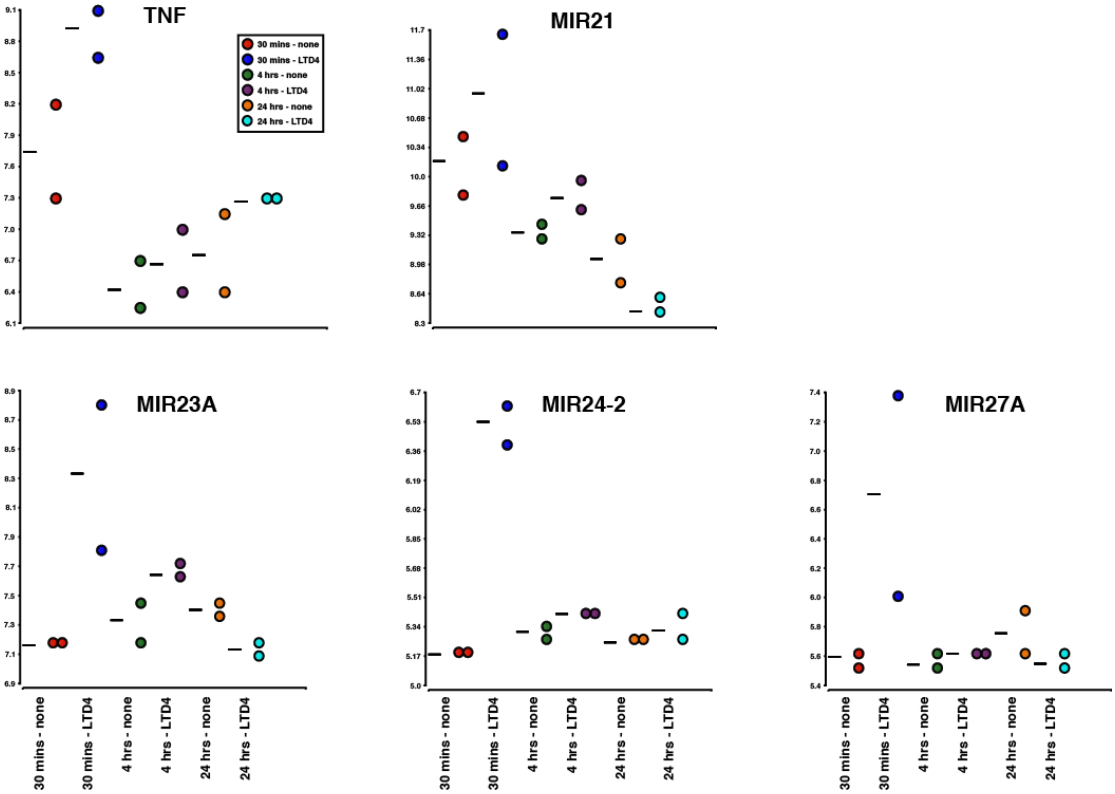


Figure 5.10 Expression profile of genes regulated by LTD_4. X-axis shows treatment and time combinations. Y-axis shows log_2 signal intensity.

5.2.3.1 EGR1/EGR2/EGR3

EGR1 was found to be differentially expressed following treatment with LTD_4 only at 30 min time-point. In comparison, 4- and 24 hour treatment of LTD_4 did not induce any change in gene expression between LTD_4-treated cells and untreated. EGR2 was also differentially expressed at 30 min time-point when treated with LTD_4, and we could still detect a very small change at 4-hour time-point. However at 24 hours, no change could be detected. EGR3 followed a similar pattern to EGR1 and the only difference in expression was after 30 minutes of treatment.

EGRs are a family of Cys2His2-type zinc finger transcription factors, which consist of four members: EGR1, EGR2, EGR3 and EGR4. In CD4+ T cells, reports show that EGR2 is associated with the induction of anergy and may play a role in the anergic state of A.E7 T cells. The expression profile of EGR2 was found to be initially upregulated in both anergised and fully activated T cells before their proliferation. EGR2 expression was found to be inhibited by cyclosporin A and found to decrease more rapidly in proliferating cells than anergized ones. Interestingly, IL-2 blocked the high expression levels of EGR2 in anergy, which therefore reversed the anergic state (Harris et al. 2004).

Using microarray analysis Safford *et al.* showed that EGR2 and EGR3 act as regulators of T cell activation, as part of a negative feedback loop in order to dampen the immune response. The group showed that in the presence of co-stimulation and IL-2 production, activation takes place and EGRs cannot act as negative regulators. However in the absence of co-stimulation, small amounts of IL-2 are produced, and EGR2 and EGR3 are able to inhibit T cell activation and promote anergy. This data

suggested that both EGR2 and EGR3 may be involved in promoting the upregulation of certain inhibitory genes (Safford et al. 2005).

EGR2 has also been reported to play a critical role in the nervous system. Indeed, mutations of the EGR2 gene prevent Schwann cell development and peripheral nerve myelination in mice and lead to the development of demyelinating neuropathy (Topilko et al. 1994). In humans, EGR2 mutations have been linked to Charcot-Marie-Tooth disease type 1, Dejerine-Sottas syndrome and congenital hypomyelination neuropathy (Shy et al. 2002).

Several recent genome-wide association studies have identified new genetic links between EGR2 and human autoimmune diseases, such as Crohn's disease (Consortium 2007; Rioux et al. 2007). A candidate gene analysis study also reported that EGR2 polymorphisms could influence the susceptibility of developing systemic lupus erythematosus (SLE) in humans. Mice with T cell-specific EGR2 cKO (conditional knockout) have also been shown to develop lupus-like autoimmunity (B. Zhu et al. 2008).

EGR1 (also called Zif268, NGF1-A, Krox24, or TIS8) is known as an immediate-early response gene, based on its very quick induction within minutes of a stimulus, and its rapid downregulation, often within hours (Yan et al. 2000a). Like the other members of the EGR family, EGR1 is a zinc finger transcription factor which can be induced by a variety of stimuli. EGR1 has been shown to regulate the expression of a large number of genes, including repair enzyme systems, angiogenic factors, cytokines and apoptotic factors (Yan et al. 2000).

In a study on COPD by Ning *et al.,* exposure to aqueous cigarette smoke extract (CSE) treatment was seen to stimulate EGR1 expression and transcriptional activity in human lung fibroblasts. The group also showed that EGR1 could modulate the activities of matrix metalloproteases MMP-2 and MMP-9 in mouse lung fibroblasts (Ning et al. 2004). The group investigated gene expression profiling studies and identified a number of inflammation-related proteins that are modulated in COPD, including adhesion molecules, and signaling molecules and transcription factors known for their roles in regulating inflammatory processes. Among the thousands of genes differentially expressed in COPD, EGR1 mRNA was one of the significantly upregulated transcripts identified by both SAGE and microarray analysis, with confirmation by RT-PCR and immunohistochemical approaches (Ning et al. 2004).

In a study by McCaffrey, a sustained expression of EGR1 was demonstrated in atherosclerosis. Using RNA from fibrous cap of carotid endarterectomy samples and cDNA expression arrays, they found a 5-fold increase in EGR1 mRNA in lesions, compared to media. Additionally, the lesions also showed an increase in known EGR1 target transcripts such as TNF-α, ICAM-1, and M-CSF. They also found EGR1 protein in atherosclerosis lesions, particularly in smooth muscle cells (McCaffrey et al. 2000).

EGR1 has also been associated with ischemia as it can trigger the expression of key regulators of inflammation, coagulation and vascular permeability. Chemokines such as IP-10 and MIP-2, and TF, PAI-1, ICAM-1 and VEGF were upregulated and this was correlated to rapid EGR1 activation. In a murine model of lung ischemia/reperfusion, deletion of the gene encoding EGR1 led to a decrease in the expression of these molecules, along with enhanced animal survival and organ function. This showed that

EGR1 regulates the activation of coagulation, leukocyte accumulation and vascular permeability in the ischemia/reperfusion lung, which have important effects on end-organ function (Yan et al. 2000b).

This suggests that the EGR family members may play a role in activating the transcription of proinflammatory mediators in Th2 cells in response to LTD_4.

5.2.3.2 NR4A2/NR4A3

NR4A2 followed a similar pattern of expression to EGR1. Differential expression was observed at 30 min after LTD_4 treatment and no other difference was observed at other time points, indicating that NRA42 is rapidly upregulated and downregulated before 4 hours. NR4A3 followed a similar pattern of expression.

NR4A2 and NR4A3 are members of the orphan nuclear NR4A receptors superfamily of transcription factors, which consists of NR4A1 (also known as Nur77), NR4A2 (Nurr1) and NR4A3 (NOR-1), which all share high homology particularly around their DNA binding domain. NR4A1, NR4A2 and NR4A3 have emerged as key transcription regulators of cytokines and growth factor actions through regulating the inflammatory response of several diseases such as rheumatoid arthritis and psoriatic arthritis. As these receptors are ligand-independent and constitutively active, they are also tightly regulated at the level of expression, post-translational modification and subcellular localization (McMorrow and Murphy 2011).

NRA4 subfamily members are aberrantly expressed in inflamed human synovial tissue, psoriatic skin, atherosclerotic lesions, lung and colorectal cancer cells (McMorrow and Murphy 2011).In activated cells, the NR4A receptor subfamily members are rapidly and

potently induced, suggesting their importance as transcriptional mediators of inflammatory signals and their function as immediate early response genes with a wide variety of physiological and pathophysiological actions (Zhao and Bruemmer 2010).

In a study by Doi *et al*, they identified NR4A2 as a gene most significantly upregulated by peripheral T cells in multiple sclerosis. The study involved the analysis of gene expression profiles of peripheral blood T cells from MS and control subjects, which revealed that 286 of 1,263 genes were differentially expressed between the two groups. Among genes upregulated in MS, NR4A2 was most significantly overexpressed with an increase ratio of 3.6-fold. RT-PCR confirmed the expression of NR4A2 in T cells from MS patients with an expression 5-fold higher than in healthy donors. The group also investigated the role of NR4A2 in T cell-mediated autoimmune diseases such as EAE. Using C57BL/6 (B6) mice and isolating T cells after EAE induction, they measured the expression levels of NR4A2 gene RT-PCR and detected NR4A2 in T cells on days 14, 21, 28, showing a maximum value on day 21, which correlated with the severity of EAE. RT-PCR also revealed expression of NR4A2 in CNS-derived T cells on day 9, correlating to early signs of EAE, and decreased gradually thereafter until day 21. To clarify whether Th1 or Th17 cells played a key role in EAE, the group analysed the CNS T cells and their ability to produce IFN-γ and IL-17 to find that an almost equal percentage of T cells produced IFN-γ and IL-17 respectively. Finally they demonstrated that NR4A2 could regulate T cell production of inflammatory cytokines IL-17 and IFN-γ. Silencing of NR4A2 by siRNA was shown to prevent the expression of these cytokines. Additionally, they found that siRNA reduced the ability of pathogenic T cells to adoptively transfer EAE (Doi et al. 2008).

Ji *et al.* found that PGE2 stimulation of the GPCR EP1 upregulated the expression of NR4A2. Using DNA microarray analysis, the group identified that treatment of HEK293 cells stably expressing the recombinant human EP1 receptor with PGE2 resulted in a strong upregulation of NR4A2. Using qPCR and immunoblotting, they determined that NR4A2 expression following the treatment of PGE2 on HEK-EP1 cells was concentration-dependent and that NR4A2 mRNA expression was observed within 1 h of treatment with PGE2 and decreased but lasted over 6 hours. NR4A2 protein expression was induced after 3 and 6 hour of treatment with PGE2. The group also found that PGE2 stimulation of the human EP1 receptor can activate NF-kB transcriptional activity by a mechanism involving the phosphorylation of IkB. Overall the study found that the activation of EP1 receptor by PGE2 lead to a cAMP-independent activation of PKA, which resulted in transcriptional upregulation of NR4A2, via CREB and NF-kB signalling (Ji et al. 2010).

Holla *et al* attempted to identify novel PGE2 target genes in colorectal carcinoma cells and reported that the immediate early gene *NR4A2* (*Nurr1*) was induced by PGE2, which in turn regulates cell death. PGE2 can be generated from arachidonic acid by either COX1 or COX2. In this study, the group showed that COX2-derived PGE2 rapidly but transiently induced NR4A2 in colorectal carcinoma cells via a cAMP/PKA-dependent pathway (Holla et al. 2006).

NR4A2 has been shown to play an important role in physiological processes such as apoptosis in lymphocytes and other cell types, but also inflammation, and is highly inducible in macrophages by inflammatory cytokines and oxidized lipids (Pei et al. 2006). Additionally, its gene expression has been shown to increase by enhanced NF-

κB and CREB-1-binding activity on the gene promoter and also by stimulation of IL-1β and TNFα, and PGE2 (McEvoy et al. 2002; E. P. Murphy et al. 2001).

Interestingly, NR4A nuclear receptors have been linked to immune diseases and inflammation through their modulation of leukocyte functions. Although not directly related to NR4A2, a study by Kagava *et al.* reported that NR4A1 AND NR4A3 were induced by CD30 stimulation during eosinophil-specific apoptosis. The results of the study suggested that CD30-activated NR4A receptors expression may regulate eosinophil apoptosis in allergic conditions such as atopic dermatitis (Hashida et al. 2007; Kagaya et al. 2005).

It is interesting that the members of the NR4A receptor family are expressed as immediate early genes in different cell types following various stimuli and it suggests that these genes must play multiple roles in biological functions. It is a possibility that NR4 genes may also play a role in activating transcription of proinflammatory mediators in Th2 cells in response to LTD_4 (Winoto and Littman 2002).

5.2.3.3 CD69

Differential gene expression for CD69 was observed at the 30-min time-point. No change was detected at 4- and 24-hour treatment.

CD69 is a type II integral membrane protein with a C-type lectin-binding domain. It belongs to the family of natural killer cell gene complex (or NKC) of cell surface receptors. In humans, the molecule is a disulfide-linked homodimer formed by the association of two subunits of 27 and 33 kDa and it is the product of a single gene located on chromosome 12 (Schnittger et al. 1993).

CD69 is known as an activation inducer molecule or very early activation antigen as it was initially detected on the surface of activated lymphocytes. However CD69 is not restricted to activated lymphocytes and is constitutively expressed on human monocytes, platelets and epidermal Langerhans cells (Atzeni et al. 2002; Marzio et al. 1999). Reports also show that CD69 can be induced *in vitro* on the surface of most hematopoietic lineages, including recently activated T and B lymphocytes (Cosulich et al. 1987; Testi et al. 1994) but also, amongst others, activated neutrophils and eosinophils (Gavioli et al. 1992; Nishikawa et al. 1992), and human monocytes (De Maria et al. 1994). Moreover, CD69 can be induced by PMA and fMLP in neutrophils, while GM-CSF, IL-3, IL-5, and IFN-γ are appropriate stimuli for eosinophils (Nopp et al. 2000).

No physiological ligand has been identified for CD69 yet. It has however been linked to the biology of hematopoietic cells and the pathogenesis of some human diseases (Testi et al. 1994). CD69 has also been reported to induce platelet aggregation (Testi et al. 1990), T cell proliferation and pro-inflammatory cytokine production, neutrophil

degranulation, and calcium flux (Gavioli et al. 1992; Santis et al. 1992; Testi et al. 1989; Testi et al. 1990) via its crosslinking by monoclonal antibodies.

In a study by *Atzeni et al*., CD69 expression was nearly undetectable on neutrophils but increased after a 60-min activation with PMA. When neutrophils were cultured with cytokines GM-CSF, IFN-α, and IFN-γ, the number of CD69-positive neutrophils significantly increased. Studies also confirmed that CD69 crosslinking by anti-CD69 mAb resulted in the induction of several cellular responses. In PMA-activated neutrophils, CD69 crosslinking by mAb induced Ca^{2+} influx, lysozyme release, and CD11B expression (Atzeni et al. 2002).

CD69 crosslinking has also been shown on T lymphocytes, causing cellular effects such as calcium signalling, but not PKC. When PKC was simultaneously activated by PMA, CD69 was seen to induce IL-2 and IFN-γ gene expression, enhancement of CD25 expression and IL-2-dependent T cell proliferation (Testi et al. 1989).

CD69 has also been associated with allergic inflammation. Indeed, a CD69 deficiency inhibits the development of asthma, as shown in a study by Miki-Hosokawa. The group investigated the role of CD69 in the pathogenesis of allergic airway inflammation using a mouse model of allergic asthma. The results showed that CD69 played a critical role in the induction of both antigen-induced eosinophilic airway inflammation and AHR. Adding anti-CD69 antibody resulted in a dramatic reduction in the extent of airway inflammation and AHR. Using CD69KO mice crossed with BALB/c mice, results showed that OVA-induced airway inflammation and AHR were reduced in CD69-deficient mice compared to wild type, as numbers of total infiltrated leukocytes and eosinophils in the BAL fluid were significantly decreased. Analysis of mRNA expression levels showed

that levels of IL-5, IL-13 and eotaxin-2 were significantly lower in BAL fluid CD4+ T cells from CD69KO mice compared to normal. Further experiments also showed that Th2 cell numbers was lower in CD69-deficient mice following OVA-sensitisation and OVA-challenge. CD69-deficient CD4 T cells were also found to have impaired migration into the asthmatic lung. The study suggested that CD69 plays a critical role in the development of allergen-induced eosinophilic inflammation and AHR by allowing for efficient migration of Th2 cells into the asthmatic lung (Miki-Hosokawa et al. 2009).

In contrast, a study by Martin *et al.* used two models of allergic disease, ovalbumin-induced allergic airway inflammation (BALB/c genetic background) and contact hypersensitivity to oxazolone (C57BL/6J genetic background) of CD69 in knockout and wild-type mice and demonstrated that CD69 negatively regulates the inflammatory response in these models. Results showed that CD69 deficiency dramatically enhanced the inflammatory response in the ovalbumin-induced asthma model of antigen-induced airway allergy. CD69 knockout mice showed exacerbated pulmonary eosinophil recruitment, high vascular cell adhesion molecule 1 expression levels in lung vasculature, and enhanced Th2 and Th17 cytokines in the bronchoalveolar space and lung tissue. In the contact hypersensitivity model, both CD69 deficiency and treatment with anti-CD69 mAb increased inflammation. Treatment with contact allergens induced enhanced Th1 and Th17 responses in CD69-deficient mice, and neutralizing anti–IL-17 antibodies reduced skin inflammation. In both experimental systems, adoptive transfer of lymph node cells from CD69 knockout mice increased the inflammatory response in recipient mice (P. Martin et al. 2010).

Although contradictory, these studies strongly suggest that CD69 plays a complex role

in lymphocyte and other immune cell migration which is interesting in relation to our evidence that LTD_4 is chemotactic for Th2 cells. Further investigation will be required to understand the role of CD69 upregulation in response to LTD_4 treatment and the effects on Th2 cell migration.

5.2.3.4 FOS/FOSB

FOSB and FOS were differentially expressed after 30 minutes of treatment with LTD_4. No change in gene expression was observed after 4 hours or 24 hours of treatment in either of the genes. The pattern of expression of the two genes was very similar.

FOS and FOSB are members of the Fos family (c-Fos, FOSB, Fra-1, and Fra-2), which combine with Jun family members (c-Jun, JunB, and Jun D) to form activator protein 1 (AP-1), a transcription factor with very potent proinflammatory effects, which dimerizes in various combinations through a region known as a leucine zipper (P. J. Barnes 2006). AP-1 may be activated via PKC and by various cytokines, including TNF-α and interleukin IL-1β, via several types of protein tyrosine kinase (PTK) and MAP kinase, which in turn activate a cascade of intracellular kinases. Certain signals have been found to rapidly increase the transcription of the FOS gene, resulting in increased synthesis of Fos protein. Other signals lead to activation of kinases that phosphorylate c-Jun, resulting in increased activation. Once again these transcription factors are likely involved in upregulating proinflammatory genes in response to LTD_4 such as CD69 or TNF-α.

5.2.3.5 MicroRNAs

MIR21 was differentially expressed at 30 minutes of LTD_4 treatment compared to untreated cells. After 4 hours of treatment, there was still a slight increase in gene

expression in LTD_4-treated cells versus untreated. At 24 hours, treatment of LTD_4 induced a decrease in MIR21 gene expression. The same pattern of gene expression was observed for MIR23A and MIR27A. However in MIR24-2, the only gene expression difference was after 30 mins of treatment with LTD_4, after which the effect decreased until 24 hours.

MicroRNAs (or MiRNAs) are short single stranded, non-coding RNAs of approximately 21–23 nucleotides in length that regulate gene expression by binding to specific mRNA targets and promoting their degradation and/or translational inhibition (Pritchard et al. 2012). They are predicted to regulate almost one third of the human genome and are essential for cellular and organism development (Mishra et al. 2008). The discovery of microRNAs as independent functional units of noncoding RNAs has changed our views about these noncoding regions. Computational predictions suggest that the total number of different miRNA sequences in humans may approach 1000 (Berezikov et al. 2005). In most organisms, there are a limited number of miRNAs compared with the number of mRNAs and proteins; for example, the human genome is believed to encode approximately 1,000 miRNAs, whereas the number of mRNAs is typically estimated at approximately 30,000 (Pritchard et al. 2012). Although little is still known about MiRNAs, they are predicted to regulate genes involved in multiple pathways such as cell death, cell proliferation, stress resistance and fat metabolism (Ambros 2003). As little is known about MIR21, MIR23, MIR24 and MIR27, which we have identified as being upregulated in our microarray studies, we focus here on reports of miRNAs in general.

Several studies have shown a central role of miRNAs in the pathogenesis of asthma with a number of studies reporting changes in the expression of several miRNAs, which have been shown to be associated with the development and/or improvement in asthma (Ariel and Upadhyay 2012; Oglesby et al. 2010).

In a study by *Williams et al.*, they compared the microRNA profile of airway biopsies from patients with mild asthma compared to those from healthy non-asthmatic volunteers. They then looked at the effect of treatment with inhaled corticosteroid therapy over 4 weeks on the microRNA profile in patients with steroid-naive mild asthma. From their experiments they found a specific miRNA expression profile in human airway biopsies from mild asthmatic patients and in the cell types commonly associated with the airways and lung. Of these, Mir-24 was one of the 28 miRNAs to be classified as highly expressed. Comparing airway biopsy samples from asthmatic patients with those of healthy individuals, the group observed that this profile was maintained in the mild asthmatic phenotype (Williams et al. 2009).

In a review by Tomankova *et al*., they report a role for miRNAs in the physiology and pathology of the lung (mainly based on mouse studies) and a specific miRNA expression profile in the lung (Tomankova et al. 2010). Several miRNAs have been shown to be involved in homeostasis and in the lung development. Of these, miR-155 has been shown that to be involved in the differentiation of naive T cells into Th1 and Th2 cells in mice (Banerjee et al. 2010). Mice deficient in bic/miR-155 were found to display increased lung remodelling, higher bronchoalveolar leukocytes and impaired T- and B-cell responses to inflammatory stimuli (Rodriguez et al. 2007). The role of the miRNAs that we have identified in Th2 cell function will require further investigation to identify potential mRNA targets for these miRNAs.

5.2.3.6 PTGS2 (COX2)

PTGS2, also known as COX-2, followed a similar pattern to all other genes described earlier, in that LTD_4 treatment was found to affect gene expression only after 30 minutes of treatment. Expression of COX-2 had returned to baseline by the 4- and 24-hour time-points.

The COX enzymes are responsible for initiating the synthesis of prostaglandins, which are involved in regulating inflammatory responses. COX enzymes are encoded by two genes; COX-1 and COX-2. Cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2), also termed prostaglandin endoperoxide H synthase-1 and -2 (PGHS-1 and -2) are key enzymes that catalyse the first step in the synthesis of prostanoids from fatty acid precursors like arachidonic acid. When arachidonic acid is released by phospholipase A2 following stimulus, it is converted to PGH_2 by COX-1 or COX-2. PGH_2 is then converted to PGE_2 by mPGES1 and other prostanoids such as PGD_2, PGF_2, PGI_2, Thromboxane A2 (Carey et al. 2003).

The enzymes share 60% homology (Smith et al, 2000) and consist of three domains; an EGF domain, a membrane-binding domain and a catalytic domain that controls both its COX and peroxidase activity. In humans COX-1 and COX-2 are homodimers of 576 and 581 amino acids, respectively, and despite their homology, are regulated independently by different systems.

COX-1 has been found to be constitutively expressed by most cell types. It serves as a housekeeping enzyme responsible for producing basal levels of prostaglandins in the endoplasmic reticulum, and also been suggested to function only at high arachidonic concentrations, such as in inflammation. COX-1 is ubiquitously expressed and needed

for homeostasis, for example in maintaining the epithelial barrier. COX-2 is an inducible enzyme and its expression is generally undetectable in most tissues under homeostatic conditions and upregulated in response to inflammatory stimuli (Carey et al. 2003).

COX-2 is expressed under the control of the pro-inflammatory transcription factor NF-kB in response to a vast range of extracellular and intracellular stimuli. It is able to function with much lower concentrations of arachidonic acid to COX-1. Both enzymes can be inhibited by non-steroidal anti-inflammatory drugs such as aspirin and ibuprofen. Aspirin inhibits COX-1 completely, while COX-2 only partially (Smith et al. 2000).

As PGE2 is generally associated with inflammatory responses and high levels are found in the sputum of asthmatic subjects versus healthy individuals, the enhancement of PGE2 is generally positively correlated to disease severity. Although the evidence for the role of COX enzymes in Th2-mediated inflammation is still contradictive, COX-2 has been linked to allergic lung inflammation and asthma due to its associated with PGE2. Some reports have suggested that PGE2 plays a role in Th2 inflammation by inhibiting the production of Th1-specific cytokines IL-2 and IFN-γ (Betz and Fox 1991)(Betz et al, 1991). However other reports have also suggested that PGE2 can inhibit Th2-specific cytokines secretion as well as the production of IgE on B cells (Khan 1995; Pene et al. 1988).

COX-2 has also been involved in the regulation of Th17 cell differentiation during allergic lung inflammation. The study by Li *et al.* demonstrated that allergic COX-2$^{-/-}$ mice showed reduced Th17 cells in the lung, BALF and lymph nodes during lung

inflammation *in vivo*, as well as reduced levels of IL-17A in blood and BALF. The group also found that Th17 differentiation of naïve CD4+ T cells from COX-2^{-}/$^{-}$ mice was impaired *in vitro* with decreased STAT3 phosphorylation and RORγt expression. Th17 differentiation could also be inhibited by COX-2 inhibitors (H. Li et al. 2011a).

COX-2 is likely to play an important role in upregulating proinflammatory lipid production in response to LTD_4.

5.2.3.7 TNF-α

TNF-α is cytokine and member of the TNF superfamily, which plays an important role in immediate host responses against pathogens. TNF-α is generally produced by LPS-activated macrophages but also by other inflammatory cells such as T cells, monocytes, dendritic cells, B cells, neutrophils, mast cells and eosinophils (Brightling et al. 2008).

TNF-α has been implicated in the pathophysiology of several inflammatory diseases such as rheumatoid arthritis and asthma (Brightling et al. 2008). It has been shown to play a role in the promotion of airway inflammation and airway hyperresponsiveness in asthmatic patients (Berry et al. 2006; Erzurum 2006), but also in airway remodelling, by stimulating the growth and maturation of fibroblasts into myofibroblasts by promoting TGF-β expression (Baluk et al. 2009). Anti-TNF-α is a known efficacious treatment for rheumatoid disease (Olsen and Stein 2004) and has been suggested as a potential treatment for severe (or refractory) asthma (Howarth et al. 2005).

TNF-α has been implicated in several asthma-related studies. For example, it was identified as a chemoattractant for neutrophils and eosinophils (Lukacs et al. 1995), but also as an enhancer of T cell responses (Scheurich et al. 1987).

In a study by Thomas *et al*., they found that inhalation of recombinant human TNF-α by normal and asthmatic patients induced an increase in airway responsiveness and neutrophil infiltration (Thomas et al. 1995; Thomas and Heywood 2002). Studies looking at the effect of TNF-α on airway hyperresponsiveness have revealed that AHR could be caused by the release of cysteinyl leukotrienes (Huber et al. 1988), as evidenced by *in vivo* studies in rats. In this study, Huber *et al*. found that infusion of TNF-α increased the rate of cysteinyl leukotrienes production, as measured by excreted LTE_4. Anti-TNF-α prevented the increased synthesis of leukotrienes.

In a study by Haneda *et al.*, they found that LTD_4 enhanced TNF-α-induced VEGF production in human monocytic leukemia THP-1 cells (Haneda et al. 2011).

TNF-α has also been found to increase the levels of COX-2 in non-transformed epithelial cells and those of 5-LO in non-transformed epithelial cells and cancer cell lines. TNF-α and LTD_4 were both observed to increase levels of the enzyme LTC4S, which is involved in the leukotrienes biosynthesis pathway, in these types of cells. These data suggested that inflammatory mediators such as TNF-α and LTD_4 could play an important role in upregulating the expression of enzymes involved in the biosynthesis of eicosanoids during inflammation (Yudina et al. 2008). This data was supported by Serio *et al*., who found that treatment of THP-1 cells and primary human monocytes with TNF-α decreased the capacity of these cells for CysLT release, as well as LTC4S gene expression. This effect was also observed in primary bone marrow-derived macrophages and eosinophilic AML14.3D10 cells (Serio et al. 2007).

In a study by Ichiyama *et al*., they found that cysteinyl leukotrienes alone could not induce the expression of MMP-9 in THP-1 cells. However, CysLTs such as LTC_4 and LTD_4

were able to enhance the effect that TNF-α has on inducing MMP-9 expression. Interestingly, this enhancement of TNF-α -induced MMP-9 was completely inhibited by $CysLT_1R$ antagonist pranlukast, suggesting a role for the $CysLT_1$ receptor (Ichiyama et al. 2007).

Overall, these data support the importance of TNF-α in inflammatory responses, particularly its close association with cysteinyl leukotrienes.

5.3 Summary

Most genes described in this chapter show differential expression after 30 minutes of treatment with LTD_4, where LTD_4 is found to induce an upregulation in the genes. This upregulation is generally already lost at 4 hours and 24 hours.

In contrast to the data by Woszczek and Uzonyi, our data suggest that human T cells signal through CysLTR1, which is partially coupled to Gαi/Gαq, in response to LTD_4 stimulation, unlike human monocytes (which couple via Gαi). Regardless of the differences in G protein coupling, these data suggest that both signalling mechanisms can induce the expression of immediate-early genes.

There were also a few differences between our data and previous data by other groups. In monocytes, Woszczek *et al.* observed an induction of FOSB and TSC22D3, which were not found to be upregulated in HUVECs. Also, no change in EGR1 expression, a gene potently induced by CysLTR2 signalling, was observed in human monocytes (Woszczek et al. 2008b). In our data EGR family members EGR1, EGR2, EGR3, but also NR4A2 and FOSB were found to be upregulated after 30 minutes. However we also found that CD69 and three microRNAs were also induced at that time point. These differences observed between these studies could be a result of differences in signalling in different cell types, as well as the potential existence of divergent pathways that are used by CysLTR1 and CysLTR2, which are yet to be found.

Collectively the microarray studies have indicated that LTD_4 does not have a profound effect on transcriptional control in human Th2 cells. However the genes identified at the 30 min time-point suggest that a cascade of transcription factors and miRNAs are induced which may in turn regulate other genes and proteins. It is particularly

interesting that increases in CD69 and TNF-α are observed and this will require further investigation by real-time RT-PCR, flow cytometry (CD69) and ELISA (TNF-α). Given the roles of each molecule in inflammatory processes it will be interesting to further study the effect of LTD_4 stimulation on their expression.

CHAPTER 6
DISCUSSION

Chapter 6 Discussion

In the studies presented here, we have shown that CYSLTR1 expression was detected in Th1 and Th2 cells in microarrays and that Th2 cells express significantly higher levels of CYSLTR1 mRNA. We then confirmed these observations using real-time quantitative RT-PCR to find that Th2 cells selectively express CYSLTR1 mRNA with 6.5-fold higher expression than human Th1 cells, and that this expression increases with differentiation. CYSLTR1 was highly expressed in Th2 resting cells and its expression was significantly downregulated by acute cell activation, a GPCR profile commonly identified in T cells, suggesting that activated T cells have an altered chemotactic potential (Sallusto et al. 1999). In our data, we found no evidence for the expression of the other known CysLT receptors, CYSLTR2, GPR17, or P2RY12, in T cells. Following confirmation that Th2 cells do express CYSLTR1 mRNA, we sought to determine a functional role for CysLTR1 in T cells. Using FLIPR calcium flux assays, we found that cysteinyl leukotrienes induced a calcium flux in both Th1 and Th2 cells, in the rank order of potency $LTD_4 > LTC_4 > LTE_4$, with much stronger calcium response observed in Th2 cells consistent with the expression levels of CYSLTR1 mRNA. This calcium response was dose-dependent and was fully inhibited with treatment using specific $CysLT_1$ receptor antagonists MK571, montelukast and zafirlukast. These results demonstrate that the selective expression of mRNA for CYSLTR1 observed in Th2 cells results in functional CysLT1R expression. Activated Th2 cells were unresponsive to LTD4 in calcium flux assays, suggesting that T cell activation might cause heterologous desensitization of $CysLT_1$ in T cells, a common feature found in GPCR biology (Tan et al. 2004).

Pre-treatment of Th2 cells with pertussis toxin, a Gαi protein inhibitor, led to a 50% decrease in calcium mobilization, suggesting a partial signaling pathway for CysLTR1 via Gαi. In order to support these results, we used cyclic AMP assays, as the Gαi subunit GPCRs can inhibit increases in intracellular cAMP levels. LTD_4 was found to induce a 50% reduction in forskolin-induced cAMP generation, and this response was dose-dependent. These data strongly suggest that CysLTR1 is partially coupled to Gαi and Gαq in human Th2 cells. The cyclic AMP response was inhibited by the antagonist MK571, demonstrating that it is CysLTR1-dependent. Both intracellular and extracellular calcium were required for calcium flux, as determined by the use of EDTA and thapsigargin. Our finding that CysLTR1 is partially coupled to Gαi and Gαq in human Th2 cells is consistent with results found in other cell types where endogenous expression of CysLTR1 has been examined. Intracellular calcium signaling was identified in human monocytes in response to cysteinyl leukotrienes in the same rank order of potency observed in our system, and this was fully inhibited using MK571. However in the human monocytes system, pre-treatment with Gαi inhibitor pertussis toxin fully inhibited calcium flux, suggesting that CysLTR1 was coupled to Gαi only (Woszczek et al. 2008b).

Although we were able to demonstrate functional expression of CYSLTR1 in Th2 cells via mRNA and calcium flux measurements we were unable to detect CysLTR1 protein expression in Th2 cells using the commercially available antibodies to CysLTR1. We attempted both Western blotting and extracellular and intracellular flow cytometry using antibodies from two manufacturers. GPCRs in general, and CysLTR1 in particular, have proven difficult to generate high quality antibodies against. There are several

possible reasons for the limited availability of high quality antibodies for GPCRs including low levels of expression, high turnover on the cell surface, limited surface epitopes and structural instability.

We went on to examine the role of CysLTR1 expression in Th2 cell migration using chemotaxis assays. LTD_4 induced migration of Th2 cells, with a peak of migration observed at 3 nM LTD_4, with a classical bell-shaped dose-response curve. This chemotaxis was dose-dependent and was inhibited when cells were treated with antagonist MK571 suggesting that CysLTs may play a role in Th2 cell recruitment and that $CysLT_1$ receptor antagonists may function to suppress Th2 cell recruitment *in vivo*. Additionally, we investigated the effect of LTD_4 on Th2 cells using LTD_4 during Th2 cell differentiation and found that LTD_4 did not seem to have any significant effect on the expression of Th2-specific cytokines by intracellular cytokine staining, and these results were supported by mRNA expression studies. Finally, using LTD_4-treated and untreated Th2 cells at various time-points on microarrays, we found that a series of genes were significantly up-regulated following 30 minutes of treatment with LTD_4. Amongst those genes, EGR family genes and NR4A2 were also upregulated in previous expression studies performed in human monocytes and HUVECs (Uzonyi et al. 2006; Woszczek et al. 2008b). Collectively these results suggest that $CysLT_1R$ is selectively expressed on human Th2 cells and that its principal role identified to date is in Th2 cell migration.

The CysLTs are potent pro-inflammatory lipid mediators with a major role in the pathogenesis of asthma, including bronchial constriction and cell trafficking, as described in earlier chapters. Recent studies have shown that CysLTs also play a critical role in the development and amplification of antigen-specific Th2 cell–mediated

inflammation, although the mechanisms by which this occurs are not fully elucidated (Kim et al. 2006). Several studies have implicated myeloid cells in this process, with both dendritic cells (Machida et al. 2004; Okunishi et al. 2004), monocytes (Thivierge et al. 2001; Woszczek et al. 2008b), but also endothelial cells (Uzonyi et al. 2006), mast cells (Di Capite and Parekh 2009), basophils (Gauvreau et al. 2005) and peripheral blood CD34+ progenitor cells (Bautz et al. 2001) recognized as CysLT target cells.

A limited number of previous studies have also suggested that CysLT1R may be expressed by T cells (Early et al. 2007; Prinz et al. 2005; Sharma et al. 2011). For example, Prinz *et al.* showed that murine T cells expressing a mutant version of the linker for activation of T cells (LAT), *LatY136F* CD4+ T cells, responded to LTD_4. However this was not reported in wild type CD4+ T cells (Prinz et al. 2005).

To our knowledge, this is therefore the first report of T cell subset–specific expression of $CysLT_1$ receptor, and our results strongly suggest that the Th2 cell should be considered a target cell for cysteinyl leukotrienes.

Because LTD_4-mediated effects on Th2 cells are sensitive to inhibition by the $CysLT_1$ receptor antagonists MK571, montelukast, and zafirlukast, Th2 cells might be a previously overlooked target of these clinically efficacious drugs. The importance of CysLTs in the development of Th2 inflammation in the lung has been demonstrated in murine models of allergic airways disease through both genetic deletion of the LTC_4 synthase gene (Kim et al. 2006) and pharmacologic blockade with $CysLT_1$ receptor antagonists (Henderson et al. 2002). Although the mechanisms by which CysLTs function in these systems are unclear, we could suggest that CysLTs may play a role in Th2 cell activation or Th2 cell recruitment to sites of inflammation.

Interestingly, since they were first introduced into the clinic, anti-leukotriene agents have been the subject of several studies that have investigated their effects. Studies have revealed that $CysLT_1$ receptor antagonists are effective in only a subset of patients, although no mechanistic explanation of this effect on efficacy has been identified. However, a recent study has suggested that the ratio between urinary LTE_4 and exhaled nitric oxide levels can have some predictive benefit in identifying children who will respond to montelukast (Rabinovitch et al. 2010).

Furthermore, novel approaches to endotyping and phenotyping have begun to allow stratification of asthma patients into different groups (Lotvall et al. 2011; Woodruff et al. 2009). Additionally, a study by Terashima *et al.* examined the factors correlated with a clinical response to antagonist pranlukast in patients with asthma to understand why not all patients show significant clinical improvements to anti-leukotriene drugs. The group found that the clinical response to pranlukast was correlated with an elevated *ex vivo* release of cysteinyl leukotriene from leukocytes (Terashima et al. 2002). They also showed that age, total white blood cell counts, percentage of eosinophils, percentage of basophils, and serum IgE showed no correlation, consistent with reports from Reiss *et al.* on LTE_4 excretion levels after exercise-induced asthma (Reiss et al. 1997). Terashima *et al.* also showed that a relatively low percentage of patients responded to pranlukast (52%), which was similar to previously reported studies (Manning et al. 1990a).

As described in earlier chapters, several sub-phenotypes of allergic asthma have been identified in allergic asthmatic patients in the population (S. E. Wenzel 2012b). Of these, Th2-associated asthma phenotypes such as late-onset allergic asthma, exercise-

induced asthma and aspirin-induced asthma can be controlled by anti-leukotriene agents (S. E. Wenzel 2012b; S. Wenzel 2012a) . The cysteinyl leukotriene pathway, which is upregulated by Th2 cytokines and present in cells associated with Th2 inflammation such as eosinophils, basophils and mast cells, (Christie et al. 1991; Cowburn et al. 1998) is upregulated in AERD (aspirin-exacerbated respiratory disease), and some studies have suggested that the AERD phenotype is linked to leukotriene pathway–related genes (Choi et al. 2004). Anti-leukotriene drugs have also been shown to suppress exercise-induced asthma (Finnerty et al. 1992; Reiss et al. 1997). There is also evidence that leukotriene receptor antagonists (LTRAs) can be used as add-on therapy to control moderate to severe asthma (Capra et al. 2006), as corticosteroids have only negligible effects on LT synthesis, whereas LTRAs can provide additional non-steroidal anti-inflammatory properties (Capra et al. 2006).

Based on our results, we suggest that cysteinyl leukotrienes may play an important role in the activation and recruitment via migration of Th2 cells to sites of inflammation during an asthmatic response and that LTRAs may be involved in targeting Th2 cells. This hypothesis could explain some of the reasons as to why anti-leukotriene agents are efficacious only on a certain subset of asthmatic patients. If LTRAs do target Th2 cells, then patients with a Th2-associated asthma phenotype may benefit from their clinical effects.

Novel approaches to endotyping and phenotyping have begun to allow stratification of patients into different groups. For example, Woodruff *et al*. used microarrays analyses of airway epithelial brushings from patients with mild-to-moderate asthma and healthy control subjects, who were classified according to their high or low expression

of IL-13. The group identified two distinct subgroups, which they called ‘‘Th2-high’’ and ‘‘Th2-low’’ asthma (Woodruff et al. 2009). Recently, it has been shown that asthmatic patients may be divided into two groups based on their level of periostin in the peripheral blood. Periostin has been identified as a gene expressed by bronchial epithelial cells in response to IL-13, and can play a role in bronchial hyperresponsiveness and inflammation. It has been suggested that periostin level in blood could potentially be a clinically useful method to discriminate patients who do not have Th2-specific inflammation. In a study by Corren *et al*., they demonstrated that treatment with anti-IL-13 (Lebrikizumab), was more effective in patients with high periostin levels than in patients with low levels, as evidenced by an increase in FEV_1 and a decline in FeNO (Corren et al. 2011). In light of our identification of a role for CysLTR1 in Th2 cell function, it would be very interesting to examine the effect of $CysLT_1$ receptor antagonists in a similarly stratified patient group. Further *in vivo* studies in animal models with conditional deletion of CYSLTR1 in T cells will be required to fully characterize the relative importance of CysLTR1 expression on Th2 cell function during asthma pathogenesis and the potential role of anti-leukotriene agents on these cells.

In conclusion, we have shown that human Th2 cells express functional CysLTR1 and that LTD_4 causes migration of Th2 cells. These findings suggest a possible explanation for the role of CysLTs in the development or amplification of allergic disease and provide insight into potential mechanisms of action of $CysLT_1$ receptor antagonists in asthmatic subjects.

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PUBLICATION

Original article

Human T_H2 cells respond to cysteinyl leukotrienes through selective expression of cysteinyl leukotriene receptor 1

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Background: Allergic asthma is characterized by reversible airway obstruction and bronchial hyperresponsiveness associated with T_H2 cell–mediated inflammation. Cysteinyl leukotrienes (CysLTs) are potent lipid mediators involved in bronchoconstriction, mucus secretion, and cell trafficking in asthmatic patients. Recent data have implicated CysLTs in the establishment and amplification of T_H2 responses in murine models, although the precise mechanisms are unresolved.
Objectives: Preliminary microarray studies suggested that human T_H2 cells might selectively express cysteinyl leukotriene receptor 1 *(CYSLTR1)* mRNA. We sought to establish whether human T_H2 cells are indeed a CysLT target cell type.
Methods: We examined the expression of *CYSLTR1* using real-time PCR in human T_H1 and T_H2 cells. We functionally assessed cysteinyl leukotriene receptor 1 protein ($CysLT_1$) expression using calcium flux, cyclic AMP, and chemotaxis assays.
Results: We show that human T_H2 cells selectively express *CYSLTR1* mRNA at high levels compared with T_H1 cells after *in vitro* differentiation from naive precursors. Human T_H2 cells are selectively responsive to CysLTs in a calcium flux assay when compared with T_H1 cells with a rank order of potency similar to that described for $CysLT_1$ (leukotriene [LT] $D_4 > LTC_4 > LTE_4$). We also show that LTD_4-induced signaling in T_H2 cells is mediated through $CysLT_1$ coupled to $G_\alpha q$ and $G_\alpha i$ proteins, and both pathways can be completely inhibited by selective $CysLT_1$ antagonists. LTD_4 is also found to possess potent chemotactic activity for T_H2 cells at low nanomolar concentrations.
Conclusions: These findings suggest a novel mechanism of action for CysLTs in the pathogenesis of asthma and provide a potential explanation for the anti-inflammatory effects of $CysLT_1$ antagonists. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: *Human, T_H1, T_H2, cysteinyl leukotrienes, chemotaxis,* CYSLTR1*, cysteinyl leukotriene receptor 1 protein, LTD_4*

Allergic asthma is characterized by reversible airway obstruction and bronchial hyperresponsiveness to otherwise innocuous environmental antigens and is associated with inflammation of the airways involving numerous immune cell types and inflammatory mediators.[1,2] In particular, the immune pathology observed is dominated by a T_H2-type response with increased expression of the classical T_H2 cytokines IL-4, IL-5, and IL-13, which might cause many of the hallmark features of allergic airways disease.[3] Central to this T_H2 immune pathology is the allergen-specific T_H2 cell, which is the predominant source of T_H2 cytokines in asthmatic subjects, although the precise mechanisms by which the T_H2 phenotype develops in asthmatic subjects remain to be determined.[4]

Cysteinyl leukotrienes (CysLTs) are potent lipid mediators involved in the pathogenesis of a wide range of chronic inflammatory and immune disorders, including asthma, allergic rhinitis, atherosclerosis, and inflammatory bowel disease.[5,6] The CysLT leukotriene (LT) C_4 is derived from arachidonic acid through cytosolic phospholipase A_2, 5-lipoxygenase, and LTC_4 synthase. LTC_4 is transported out of the cell and converted sequentially to LTD_4 and LTE_4 by the ubiquitous enzymes γ-glutamyl transpeptidase and aminopeptidase, respectively. The biological activities of the CysLTs are mediated through at least 4 G protein–coupled receptors, cysteinyl leukotriene receptor 1 protein ($CysLT_1$), CysLT receptor 2 protein, G protein-coupled receptor 17 (GPR17), and purinergic receptor $P2Y_{12}$ protein ($P2Y_{12}$), although the role of each of these receptors in CysLT action is incompletely understood.[6-8] In asthmatic subjects the CysLTs are believed to be involved in bronchoconstriction, mucus secretion, and cell trafficking and have been shown to be chemotactic agents for eosinophils, hematopoietic progenitor cells, and monocytes.[9-11] In addition to their role in bronchoconstriction and innate cell recruitment to the lungs of asthmatic subjects, CysLTs play a role in the establishment and amplification of antigen-specific T_H2 cell–dependent immune responses in murine models of allergic airways disease.[12] Although the precise mechanistic role of CysLTs in the development of the adaptive T_H2 response is unclear, prior studies have focused on the role of CysLTs in pulmonary dendritic cell migration and maturation.[13-15] Here we

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Abbreviations used
cAMP: Cyclic AMP
CysLT: Cysteinyl leukotriene
$CysLT_1$: Cysteinyl leukotriene receptor 1 protein
CYSLTR1: Cysteinyl leukotriene receptor 1 gene/mRNA
GPR17: G protein-coupled receptor 17
LT: Leukotriene
P2RY12: Purinergic receptor $P2Y_{12}$ gene/mRNA
$P2Y_{12}$: Purinergic receptor $P2Y_{12}$

report that human T_H2 cells selectively express functional $CysLT_1$ and that LTD_4 is a potent chemotactic agonist for T_H2 cells. T_H2 cells exhibit robust calcium signaling in response to all 3 CysLTs, with the most potent response observed with LTD_4 followed by LTC_4 and LTE_4, which is consistent with previous studies on $CysLT_1$.[16,17] The calcium signaling in response to LTD_4 was completely blocked with the selective $CysLT_1$ antagonists MK571, montelukast, and zafirlukast. These findings suggest a novel mechanism of action for CysLTs in the pathogenesis of asthma and provide a potential explanation for the anti-inflammatory effects of $CysLT_1$ antagonists.

METHODS

Reagents

Leukotrienes (LTC_4, LTD_4, and LTE_4) and $CysLT_1$ antagonists (MK571, montelukast, and zafirlukast) were purchased from Cayman Chemical (Ann Arbor, Mich). Recombinant chemokines (CXCL12 and CCL18) were purchased from R&D Systems (Abingdon, United Kingdom). Pertussis toxin, thapsigargin, and forskolin were purchased from Sigma-Aldrich (Dorset, United Kingdom). EDTA was purchased from Life Technologies (Paisley, United Kingdom).

Human T-cell isolation and differentiation

These studies were approved by the Research Ethics Committee of Guy's Hospital with informed consent. The donors used in this study were nonatopic, nonasthmatic male subjects (25-40 years) with no other chronic or acute illnesses at the time of venipuncture. Naive $CD4^+$ T cells were isolated from peripheral blood by using magnetic positive selection of $CD4^+$ cells (Dynal CD4 Positive Isolation kit; Invitrogen, Paisley, United Kingdom) followed by depletion of $CD45RO^+$ memory cells, as described in detail previously.[18] Naive T cells were stimulated with anti-CD3 (clone OKT3, ECACC, prepared in house) and anti-CD28 (clone 15E8; Sanquin Reagents, Amsterdam, The Netherlands) in the presence of cocktails of cytokines and antibodies to generate T_H1 and T_H2 cells, as described in detail previously.[18] Every 7 days, T_H1/T_H2 differentiation was assessed by using intracellular cytokine staining, as described previously.[18]

RNA isolation, microarrays, and real-time reverse transcription PCR

Cells for RNA isolation were snap-frozen in liquid nitrogen and stored at −80°C. Total cellular RNA was isolated with the miRNeasy mini kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions. cRNA samples were prepared for microarray hybridization to GeneChip U133 plus 2 arrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, Calif). Fragmented cRNA was hybridized to GeneChip arrays at 45°C for 18 hours. Arrays were washed and stained with streptavidin-phycoerythrin, according to the manufacturer's instructions, on the GeneChip fluidics station 450 (Affymetrix). Fluorescent signals were detected with the GeneChip scanner 3000. Images were analyzed with the GeneChip operating software (Affymetrix) to generate raw data as .cel files. Further analysis was performed with the Partek Genomics Suite (Partek, St Louis, Mo) by using the gene expression workflow to identify differentially expressed genes. Briefly, robust multichip average preprocessing was performed, and genes differentially expressed between T_H1 and T_H2 cells were identified by using the Partek ANOVA model. One thousand seven hundred ninety-four genes were identified as differentially expressed with a P value of less than .05, including Benjamini-Hochberg step-up for false discovery rate. Data shown in Table I are the mean probe-set signals from 3 independent biological replicates for each condition. cDNA synthesis and real-time PCR were performed with TaqMan MGB probes (Applied Biosystems, Invitrogen, Paisley, United Kingdom), as described in detail previously.[19] TaqMan probe sets used in this study were as follows: cysteinyl leukotriene receptor 1 *(CYSLTR1)*, Hs00272624_s1; cysteinyl leukotriene receptor 2 *(CYSLTR2)*, Hs00272624_s1; *IL5*, Hs00174200_m1; *IFNG*, Hs00174143_m1; and *18s* rRNA, 4319413E. Statistical analyses were performed by comparing gene expression levels relative to *18s* rRNA levels by using a 2-way repeated-measures ANOVA with Bonferroni post tests in GraphPad Prism version 5.0a software (GraphPad, Inc, La Jolla, Calif).

Calcium-signaling assay

Cultured human T_H1 and T_H2 cells were tested for calcium signaling when fully differentiated at days 21 and 28 in response to LTB_4, LTC_4, LTD_4, and LTE_4. Cells were resuspended at 200,000 cells/well in 100 μL of RPMI plus 20 mmol/L HEPES and plated onto a 96-well, black-wall, clear, flat-bottom assay plate (Costar), and an equal volume of loading buffer (Component A with 1X HBSS Buffer; FLIPR Calcium 4 Assay Kit; Molecular Devices, Eugene, Ore) was added. The plate was incubated for 45 minutes at 37°C and 5% CO_2. After incubation, the plate was centrifuged at 200 *g* for 5 minutes and transferred directly to a FlexStation 3 Microplate Reader (Molecular Devices) at 37°C. Varying concentrations of CysLTs were added as indicated. Where indicated, the selective $CysLT_1$ antagonist MK571 (100 nmol/L), montelukast (100 nmol/L), or zafirlukast (100 nmol/L) was added 5 minutes before adding ligand. In some experiments pertussis toxin (100 ng/mL) was added 16 hours before the assay was performed to inhibit $G_\alpha i$. Where indicated, thapsigargin (1 μmol/L) or EDTA (2.5 mmol/L) was added 5 minutes before adding ligand. Results were analyzed with SoftMax Pro Software (Molecular Devices), and data are shown as a percentage of maximal response. Controls included a negative medium control of RPMI plus 20 mmol/L HEPES and a positive control of stromal cell–derived factor 1/CXCL12, and all experiments were performed with triplicate wells.

Cyclic AMP assay

Intracellular cyclic AMP (cAMP) levels were measured by luminescence with the HitHunter cAMP XS+ assay (DiscoverRX, Birmingham, United Kingdom). The assay was carried out according to the manufacturer's protocol for a 3-reagent addition. T_H2 cells were suspended in PBS at a concentration of 10×10^6/mL and warmed to 37°C, and 1 nmol/L 3-isobutyl-1-methylxanthine (Sigma) was added to inhibit cAMP phosphodiesterases. The T_H2 cell suspension (2×10^5 cells/well) was loaded into a 96-well plate, and 20 μmol/L forskolin was added to stimulate cAMP induction along with varying concentrations of LTD_4, as indicated. Where indicated, 100 nmol/L MK571 was added 5 minutes before adding ligand. Plates were incubated at 37°C for 15 minutes before cells were processed according to the kit's instructions, and cAMP signal was detected by means of luminescence measured 4 hours after cell lysis. Luminescence was detected by using a Flexstation 3 (Molecular Devices), and analysis of cAMP concentrations was performed with SOFTMax Pro software (Molecular Devices). Data are shown as a percentage of maximal response.

Chemotaxis assay

Chemotaxis assays were carried out by using a modification of a protocol[20] obtained from Andrew Luster (Massachusetts General Hospital, Harvard, Boston, Mass) by using 24-well 6.5-mm Transwell plates with a polycarbonate membrane insert filter and a pore size of 5.0 μm (3421; Corning, Corning, NY). *In vitro* cultured human T_H2 cells were resuspended at 1×10^6/mL in RPMI buffer; rested for 4 hours in a low rIL-2 concentration (25 U/mL;

TABLE I. Expression of selected genes in preliminary microarray analysis

Gene name	Probe set ID	T_H1, resting	T_H1, activated	T_H2, resting	T_H2, activated
CYSLTR1	231747_at	349.4	179.2	1053	101.1
CYSLTR2	220813_at	6.549	6.517	10.02	7.413
GPR17	215225_s_at	3.993	4.112	3.956	4.067
P2RY12	224102_at	4.214	4.363	4.16	4.279
PGK1	200737_at	1902	1669	1956	1605
TAF9B	221618_s_at	206.6	74.86	312.6	137.3
ZCCHC5	1552935_at	6.926	9.382	6.134	6.99

Novartis, Horsham, United Kingdom); and then harvested, washed in PBS, and resuspended at 500,000 cells/100 μL in chemotaxis buffer (RPMI + 20 mmol/L HEPES + 0.5% ultrapure BSA [Sigma, A7638]). Six hundred microliters of chemotaxis buffer with or without the chemokine or leukotriene of interest was loaded on the bottom of the Transwell. Each chemotactic agent (LTD_4, LTE_4, and the positive control stromal cell–derived factor 1) was tested in duplicate. By using tweezers, the Transwell filter was placed in the bottom well, and 100 μL of cells was added and left to migrate for 2 hours in an incubator at 37°C 5% CO_2. After 2 hours, the plate was placed at 4°C for 15 minutes. Migrated cells were counted by using flow cytometry, with polystyrene beads as a reference (polystyrene 15-μm beads; Polysciences, Warrington, Pa). A chemotactic index was calculated as the ratio of migration to chemoattractant compared with the basal migration to buffer alone.

RESULTS

Human T_H2 cells selectively express *CYSLTR1*

We have previously reported a method to generate highly polarized human T_H1 and T_H2 cells from naive precursors *in vitro* and have used this model extensively to investigate the lineage-specific functions of these cells.[18,19,21] To identify novel lineage-specific molecules that might play a selective role in T_H2 cell function, we have previously performed a preliminary microarray-based study comparing T_H1 and T_H2 cells. Analysis of CysLT receptor mRNA expression in these samples revealed that *CYSLTR1* mRNA was highly and selectively expressed by T_H2 cells when compared with T_H1 cells ($P = .000692$, Table I). Expression of other potential CysLT receptors, *CYSLTR2*, *GPR17*, and *P2RY12*, was undetectable by means of microarray in both T_H1 and T_H2 populations (Table I). Intracellular cytokine staining of resting and activated T_H1 and T_H2 cells demonstrated that highly polarized T_H1/T_H2 populations were generated (Fig 1, *A*). On activation, at least 99% of the T_H1 cells express IFN-γ with minimal T_H2 cytokine expression. In contrast, the T_H2 cells express negligible IFN-γ, and a very high proportion express T_H2 cytokines (IL-4, 42%; IL-5, 46%; and IL-13, 85%). Quantitative real-time RT-PCR of highly differentiated T_H1 and T_H2 samples confirmed that the T_H1 and T_H2 cells express high levels of transcripts for the hallmark cytokines IFN-γ (T_H1) and IL-4, IL-5, and IL-13 (T_H2) on acute activation (Fig 1, *B*, and data not shown). Examination of *CYSLTR1* mRNA expression by using real-time RT-PCR confirmed that it is selectively expressed by resting T_H2 cells with 6.5-fold higher levels than seen in resting T_H1 cells (Fig 1, *C*). The selective expression of *CYSLTR1* was predominantly observed in cells before activation, with a substantial decrease in mRNA expression after acute activation of T_H2 cells (Fig 1, *C*). Real-time PCR analysis also confirmed that *CYSLTR2* mRNA was not expressed at high levels in either T_H1 or T_H2 cells (Fig 1, *C*). Examination of *CYSLTR1* mRNA expression during the time course of differentiation revealed that expression increased substantially during T_H2 differentiation (Fig 1, *D*), suggesting that *CYSLTR1* expression is acquired during T_H2 differentiation rather than being expressed on naive T cells and selectively lost during T_H1 differentiation. This also indicated that expression of *CYSLTR1* by T_H2 cells is not merely caused by exposure to IL-4 because it requires at least 3 weeks of *in vitro* culture before high levels of expression are observed (Fig 1, *D*). The genes adjacent to *CYSLTR1* (ie, *PGK1*, *TAF9B*, and *ZCCHC5*) were not differentially expressed between T_H1 and T_H2 cells, suggesting that the T_H2-selective expression of *CYSLTR1* is not merely due to the gene being located in close proximity to other T_H2-specific genes (Table I).

T_H2 cell–specific calcium flux in response to CysLTs

CysLT signaling through $CysLT_1$ induces calcium flux in other cell types known to express the receptor. *In vitro* differentiated T_H1 and T_H2 cells were treated with LTD_4 and intracellular calcium responses were measured to determine whether the selective expression of *CYSLTR1* mRNA observed in T_H2 cells resulted in functional expression of $CysLT_1$ in these cells (Fig 2, *A*). LTD_4 induced a substantial calcium mobilization in T_H2 cells at concentrations similar to those reported for $CysLT_1$ in other systems (median effective concentration, approximately 1 nmol/L). A much weaker response to LTD_4 was observed in T_H1 cells at higher concentrations. This confirmed that human T_H2 cells selectively express a functional receptor for LTD_4. Treatment of T_H2 cells with LTC_4, LTD_4, and LTE_4 showed that all 3 CysLTs induced calcium mobilization, with LTD_4 being the most potent, followed by LTC_4 and LTE_4 (Fig 2, *B*). This rank order of potency ($LTD_4 > LTC_4 > LTE_4$) resembles the results of previous studies on $CysLT_1$ in other cell types, suggesting that T_H2 cells express functional $CysLT_1$.[16,17] Interestingly, in contrast to the original studies with transfectants expressing $CysLT_1$, the potency of LTC_4 and LTE_4 is similar until higher concentrations of agonist are used (10-100 nmol/L), where LTC_4 induces more calcium flux. The $CysLT_1$ selective antagonists MK571 (100 nmol/L), montelukast (100 nmol/L), and zafirlukast (100 nmol/L) were used to treat T_H2 cells before treatment with 100 nmol/L LTD_4 (Fig 2, *C*) to confirm the identity of the CysLT-responsive receptor expressed by T_H2 cells. All 3 $CysLT_1$ antagonists almost completely inhibited calcium mobilization, confirming that the calcium flux in response to LTD_4 by human T_H2 cells is dependent on selective expression of functional $CysLT_1$.

$CysLT_1$ is coupled to both $G_\alpha i$ and $G_\alpha q$ G proteins in human T_H2 cells

T_H2 cells were preincubated with the $G_\alpha i$ inhibitor pertussis toxin and treated with increasing concentrations of LTD_4 to further characterize the signaling mechanisms involved in

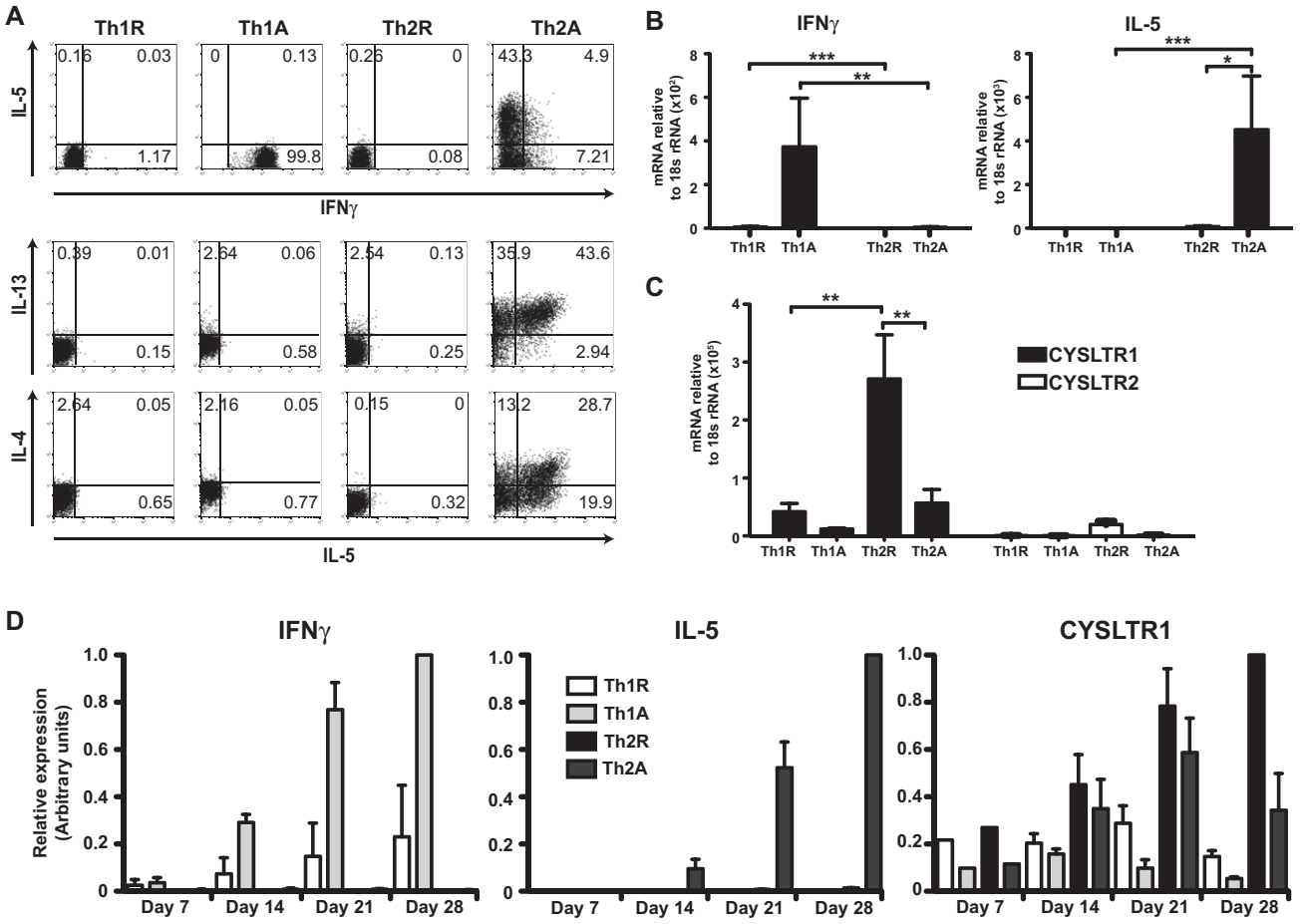

FIG 1. T_H2 cells selectively express *CYSLTR1*. **A,** Intracellular cytokine staining of human T_H1 and T_H2 cells. Cells were either resting or activated for 4 hours with phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (500 ng/mL). **B** and **C,** Real-time RT-PCR analysis of T_H1 and T_H2 cells for IFN-γ and IL-5 (Fig 1, *B*) and *CYSLTR1* and *CYSLTR2* (Fig 1, *C*). **D,** Time-course analysis of gene expression during T_H1/T_H2 differentiation by using real-time RT-PCR. Data shown are representative of 5 experiments with different donors except Fig 1, *B*, *C*, and *D*, which show means ± SEMs of 5 experiments. $*P < .05$, $**P < .01$, and $***P < .001$, 2-way ANOVA with Bonferroni post test. *A*, Activated; *R*, resting.

CysLT-induced calcium flux (Fig 3, *A*). Pertussis toxin partially inhibited calcium mobilization in response to LTD_4, with approximately 50% inhibition of calcium flux at the highest LTD_4 concentration, suggesting that $CysLT_1$ is coupled to both $G_\alpha i$ and $G_\alpha q$ G proteins in T_H2 cells. To confirm a role for $G_\alpha i$ in $CysLT_1$ signaling, we examined whether LTD_4 could inhibit cAMP signaling in T_H2 cells. Treatment of T_H2 cells with LTD_4 alone did not induce cAMP in a luminescence-based cAMP assay (Fig 3, *B*). Treatment of T_H2 cells with forskolin induced a robust increase in cAMP levels, which was partially inhibited (approximately 50%) by LTD_4 in a concentration-dependent manner (Fig 3, *B*). Pretreatment of the cells with the $CysLT_1$ selective antagonist MK571 completely blocked the LTD_4-dependent inhibition of cAMP signaling, confirming that $CysLT_1$ is partially coupled to $G_\alpha i$ in T_H2 cells (Fig 3, *C*). Preincubation of T_H2 cells with thapsigargin to deplete intracellular endoplasmic reticular calcium stores completely inhibited calcium mobilization in response to LTD_4, and chelation of extracellular calcium with EDTA partially inhibited calcium flux (Fig 3, *D*). These data suggest that LTD_4 signaling through $CysLT_1$ activates store-operated calcium channels in T_H2 cells.

LTD_4 induces chemotaxis of human T_H2 cells

CysLTs have previously been shown to be chemotactic agents for eosinophils, hematopoietic progenitor cells, and monocytes.[9-11] To examine whether LTD_4 treatment causes chemotaxis of human T_H2 cells, we performed a Transwell-based chemotaxis assay.[20] The chemokine CXCL12 is strongly chemotactic for human lymphocytes[22] and caused a robust chemotactic response in human T_H2 cells (Fig 4). In contrast, CCL18, which has been reported to be selectively chemotactic for human T_H2 cells,[23] did not cause chemotaxis (Fig 4). This might be due to differences in polarization protocols because in the previous study only a single round of *in vitro* polarization was performed. LTD_4 induced concentration-dependent chemotaxis of human T_H2 cells with a classical bell-shaped curve (Fig 4). Maximal chemotaxis was observed at 3 nmol/L LTD_4, which is very similar to the median effective concentration for LTD_4 observed in the calcium mobilization studies.

DISCUSSION

The CysLTs are potent proinflammatory lipid mediators with a major role in the pathogenesis of asthma, including bronchial

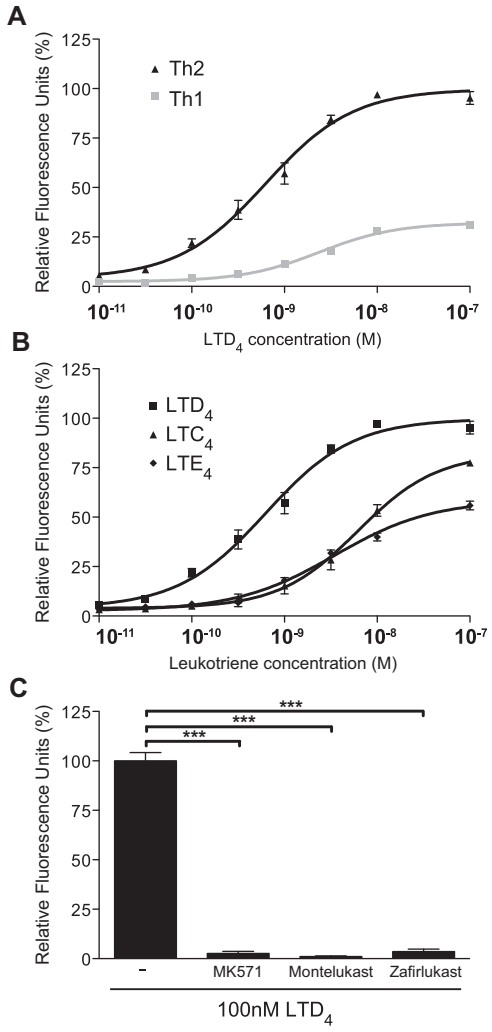

FIG 2. T_H2 cells express functional $CysLT_1$. **A,** Calcium flux in response to increasing concentrations of LTD_4 in T_H1 and T_H2 cells. **B,** Calcium flux in T_H2 cells in response to increasing concentrations of LTC_4, LTD_4, and LTE_4. **C,** Inhibition of calcium flux in T_H2 cells in response to 100 nmol/L LTD_4 by 3 different $CysLT_1$ antagonists: MK571, montelukast, and zafirlukast. Data are presented as mean ± SEM percentages of maximum response to LTD_4 from 3 experiments with different donors. ***P < .001, 1-way ANOVA with Tukey post test.

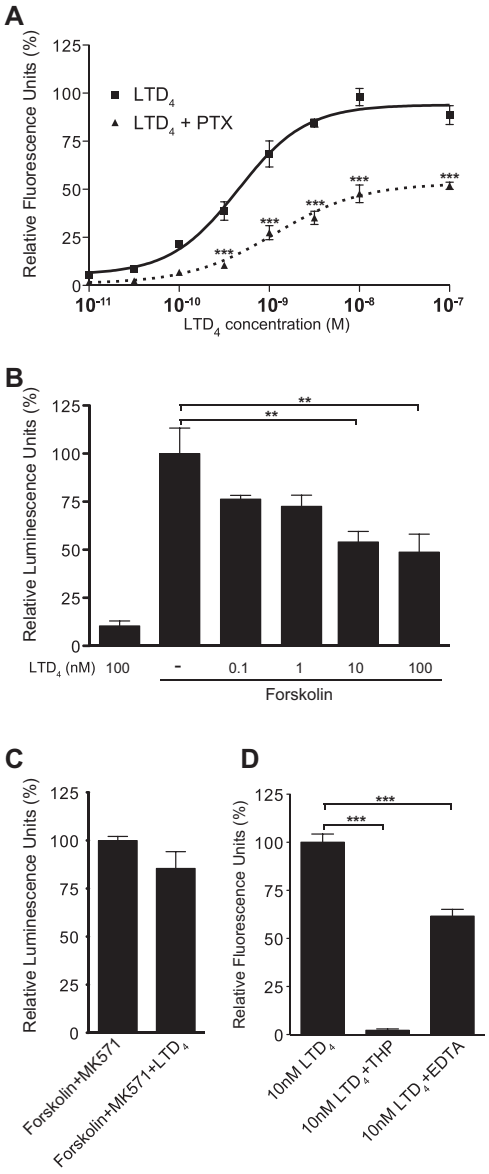

FIG 3. $CysLT_1$ is partially G_i and G_q coupled in T_H2 cells. **A,** Pertussis toxin *(PTX)*–mediated inhibition of calcium response to LTD_4 in T_H2 cells. ***P < .001, 2-way ANOVA with Bonferroni post test. **B,** LTD_4-mediated inhibition of cAMP signaling in T_H2 cells. Cells were treated with forskolin to induce cAMP, and increasing concentrations of LTD_4 inhibited cAMP generation. **C,** The $CysLT_1$ antagonist MK571 blocks the LTD_4-mediated inhibition of cAMP response. Cells were treated with 100 nmol/L LTD_4. **D,** Thapsigargin *(THP)*–mediated inhibition and EDTA-mediated partial inhibition of calcium flux response to LTD_4 in T_H2 cells. All data are expressed as mean ± SEM percentages of maximum response to LTD_4 or forskolin from 3 experiments with different donors. Fig 3, *B* and *D*: **P < .01 and ***P < .001, 1-way ANOVA with Tukey post test.

constriction and cell trafficking. Recent studies have shown that CysLTs also play a critical role in the development and amplification of antigen-specific T_H2 cell–mediated inflammation, although the mechanisms by which this occurs are not fully elucidated.[12] Several studies have implicated myeloid cells in this process, with both dendritic cells[13,14] and monocytes[11] recognized as CysLT target cells. CysLTs have been shown to potentiate dendritic cell functions, such as antigen presentation and cytokine production, in 2 murine models of allergic airway disease.[13,14] Moreover, the $CysLT_1$ receptor antagonist pranlukast was shown to inhibit antigen-dependent eosinophilic airway inflammation, mucus production, and airway hyperresponsiveness in an inhalation model of allergic airways disease.[13] We have previously shown that human monocytes are activated by LTD_4, which induces expression of several immediate early genes and causes chemotaxis.[11]

Here we show, for the first time, that human T_H2 cells selectively express *CYSLTR1* mRNA with 6.5-fold higher expression than human T_H1 cells (Fig 1, *C*). We found no evidence for expression of the other known CysLT receptors, *CYSLTR2*, *GPR17*, or *P2RY12*, in T cells. T_H2 cells selectively responded to CysLTs through calcium signaling in a rank order that resembled, to some extent, previous work on $CysLT_1$, with LTD_4 being most potent, followed by LTC_4 and LTE_4

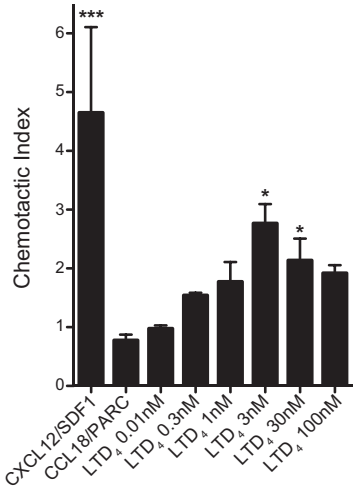

FIG 4. LTD_4 is chemotactic for T_H2 cells. Chemotactic agents were assessed in a Transwell-based system. Cells were allowed to migrate for 2 hours. Varying concentrations of LTD_4 were used as shown. Control chemokines CXCL12 and CCL18 were used at 10 nmol/L concentration. Data shown are means ± SEMs of 3 independent experiments with different donors. ***$P < .001$ and *$P < .05$, 2-way ANOVA with Bonferroni post test.

(Fig 2, *B*). Calcium flux in response to LTD_4 was completely inhibited by treatment with the $CysLT_1$ selective receptor antagonists MK571, montelukast, or zafirlukast (Fig 2, *C*). These results demonstrate that the selective expression of mRNA for *CYSLTR1* observed in T_H2 cells results in functional $CysLT_1$ expression. *CYSLTR1* mRNA was significantly downregulated by acute activation of T_H2 cells (Fig 1, *C*), and activated T_H2 cells were unresponsive to LTD_4 in calcium flux assays (data not shown), suggesting that T-cell activation might cause heterologous desensitization of $CysLT_1$ in T cells. This is a common feature of several G protein–coupled receptor in T cells, most notably the chemokine receptors, and it suggests that activated T cells have an altered chemotactic potential.[24] Treatment of T_H2 cells with the $G_\alpha i$ selective inhibitor pertussis toxin resulted in a 50% inhibition of calcium mobilization, suggesting that $CysLT_1$ was partially coupled to $G_\alpha i$ in T_H2 cells (Fig 3, *A*). $G_\alpha i$ G protein–coupled receptors can inhibit increases in intracellular cAMP levels. Treatment of T_H2 cells with LTD_4 partially (50%) inhibited forskolin-induced cAMP generation (Fig 3, *B*). The LTD_4-dependent inhibition of cAMP signaling could be completely blocked by MK571, demonstrating that it is $CysLT_1$ dependent (Fig 3, *C*). Furthermore, LTD_4-induced calcium mobilization is dependent on store-operated calcium channels because it is completely inhibited by thapsigargin (Fig 3, *D*). Collectively, these data suggest that $CysLT_1$ is coupled to both $G_\alpha i$ and $G_\alpha q$ G proteins in T_H2 cells. We have also shown that LTD_4 is chemotactic for human T_H2 cells (Fig 4). The peak of migration was observed at 3 nmol/L, with a classical bell-shaped dose-response curve.

A limited number of previous studies have suggested that $CysLT_1$ might be expressed by T cells.[25-27] Prinz et al[25] showed that murine T cells expressing a mutant version of the linker for activation of T-cells (LAT) adaptor molecule express *Cysltr1* and chemotax in response to LTD_4. Early et al[26] showed that short-term treatment with IL-4 caused an increase in *CYSLTR1* mRNA in T cells. Sharma et al[27] recently found evidence for upregulation of *Cysltr1* mRNA in T cells in a compound murine mutant in which the *IL2* gene was deleted in a forkhead box protein 3 mutant strain. However, to our knowledge, the data presented here are the first report of T cell subset–specific expression of any CysLT receptor, and the results strongly suggest that the T_H2 cell should be considered a CysLT target cell. Because LTD_4-mediated effects on T_H2 cells are sensitive to inhibition by the $CysLT_1$ receptor antagonists MK571, montelukast, and zafirlukast, T_H2 cells might be a previously overlooked target of these clinically efficacious drugs. The importance of CysLTs in the development of T_H2 inflammation in the lung has been demonstrated in murine models of allergic airways disease through both genetic deletion of the LTC_4 synthase gene[12] and pharmacologic blockade with $CysLT_1$ receptor antagonists.[28] Although the mechanisms by which CysLTs function in these systems are unclear, it is tempting to speculate that a direct role in T_H2 cell activation or migration might be involved. Further *in vivo* studies in animal models with conditional deletion of *CYSLTR1* in T cells will be required to fully characterize the relative importance of $CysLT_1$ expression on T_H2 cell function during asthma pathogenesis.

It has been clear for many years that there are several subphenotypes of allergic asthmatic patients in the population. Since they were first introduced into the clinic, it has been recognized that the $CysLT_1$ antagonists are effective in only a subset of patients, although no mechanistic explanation of this phenomenon has been identified.[29] However, a recent study has suggested that the ratio between urinary LTE_4 and exhaled nitric oxide levels can have some predictive benefit in identifying children who will respond to montelukast.[30] Furthermore, novel approaches to endotyping and phenotyping have begun to allow stratification of patients into different groups.[31-33] A striking example of this is the recent demonstration that asthmatic patients can be stratified into 2 groups based on the level of periostin in the peripheral blood.[34] Periostin has been identified as a gene expressed by bronchial epithelial cells in response to IL-13.[33] The authors contend that the periostin level in blood is a clinically useful method to discriminate patients with classical T_H2-driven allergic asthma and patients who do not have such inflammation. In an elegant study they demonstrate that anti–IL-13 might have some benefit in patients with high periostin levels.[34] In light of our identification of a role for $CysLT_1$ in T_H2 cell function, it would be very interesting to examine the effect of $CysLT_1$ antagonists in a similarly stratified patient group.

In conclusion, we have shown that human T_H2 cells express functional $CysLT_1$ and that LTD_4 causes chemotaxis of T_H2 cells. These findings suggest a possible explanation for the role of CysLTs in the development or amplification of allergic disease and provide insight into potential mechanisms of action of CysLT receptor antagonists in asthmatic subjects.

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Key messages

- **Human T_H2 cells selectively express *CYSLTR1* mRNA.**
- **Functional analysis shows that T_H2 cells respond to CysLTs.**
- **LTD_4 is chemotactic for T_H2 cells.**

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